Advances in Delivery Science and Technology

Padma V. Devarajan
Sanyog Jain *Editors*

Targeted Drug Delivery: Concepts and Design

CRS Leading Delivery Science and Technology

Springer

Advances in Delivery Science and Technology

Series Editor
Michael J. Rathbone

More information about this series at http://www.springer.com/series/8875

Padma V. Devarajan • Sanyog Jain
Editors

Targeted Drug Delivery: Concepts and Design

Springer

Editors
Padma V. Devarajan
Department of Pharmaceutical
Sciences and Technology
Institute of Chemical Technology
Mumbai, India

Sanyog Jain
Centre for Pharmaceutical Nanotechnology
Department of Pharmaceutics
National Institute of Pharmaceutical
Education and Research (NIPER)
Mohali, Punjab, India

ISSN 2192-6204 ISSN 2192-6212 (electronic)
ISBN 978-3-319-11354-8 ISBN 978-3-319-11355-5 (eBook)
DOI 10.1007/978-3-319-11355-5
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014955161

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Advances in drug delivery strategies have been phenomenal throughout the past few decades. The discovery of new drugs has been keenly matched by developments to ensure their effective delivery. Often, drugs considered hopeless have been revived through the design of drug delivery systems. Optimizing drug dosage regimens based on understanding the disease condition, developing patient-friendly systems to address compliance and other innovative approaches is the order of the day. Nevertheless the same has been challenged arduously by the development of resistance, particularly in cancer and infectious diseases. The threat today is the development of not just multidrug resistance but total drug resistance, which could spell impending doom.

Targeted drug delivery presents an optimal strategy to tackle such challenges. From ensuring high drug localization at the sites of action, and hence improved therapy, to limiting drug toxicity in other organs, targeted drug delivery presents a host of opportunities to revolutionize medicine. Such delivery relies heavily on nano drug delivery systems and presents manifold opportunities. Nevertheless, targeted drug delivery using nanosystems is fraught with numerous challenges.

The objective of this book is to serve as a complete reference guide for targeted drug delivery and as a ready reference for all aspects related to the theme. The book has been structured into eight sections to address the need of beginners and established researchers. Part I is an overview of the basic principles of drug targeting and possible applications therein. Part II covers the important subject of disease-based targeting with a focus on cancer and infectious diseases. Part III and IV discuss in sequence-relevant aspects related to organ-based and organelle-based targeting.

Physicochemical approaches exploited for targeting are elucidated in Part V. This includes different stimuli-responsive approaches including magnetic, thermal, and pH-dependent strategies. Prodrug-based conjugates and conjugates with polymers and lipids are also highlighted in the same section. Carrier-based approaches follow in Part VI. This part details applications of various types of nanocarriers in targeted drug delivery such as functionalized lipidic carriers, inorganic nanocarriers, and carbon nanotubes, to name a few. A study of nanocarriers in targeted delivery is incomplete without characterization techniques, addressed in Part VII. The major

challenges in the commercial success of targeted delivery systems are the regulatory hurdles and the toxicity-related issues. This is discussed in the last part of the book, Part VIII.

The book is the amalgamation of the experience and expertise of all the contributors in the field of targeted drug delivery. It is an exhaustive compilation of the multi-faceted arena of targeted drug delivery, ranging from conceptualization to product development and design and also to aspects of commercialization. Young researchers who plan to initiate research in this important field would find this book extremely relevant and handy. The book would also cater to the needs of advanced researchers in the field.

The editors also take the opportunity to express their gratitude to all the contributors for their support. Special thanks are due to Prof. Michael J. Rathbone. Editors are thankful for the valuable assistance received from Dr. (Ms) Anisha D'Souza, Mr. Ashish Kumar Agrawal, and Mr. Kaushik Thanki.

Finally, we wish to conclude by saying that this has been a true learning experience.

Mumbai, India — Padma V. Devarajan
Mohali, India — Sanyog Jain

Contents

List of Contributors

Ashish Kumar Agrawal Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Udita Agrawal Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. H.S. Gour Vishwavidyalaya, Sagar, Madhya Pradesh, India

Mansoor M. Amiji Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Boston, MA, USA

Shashwat Banerjee Actorius Innovations and Research (AIR), Pune, India

Sumati Bhatia Institut für Chemie und Biochemie, Freie Universität Berlin, Berlin, Germany

Luis M. Bimbo Division of Pharmaceutical Technology, University of Helsinki, Helsinki, Finland

Diane J. Burgess School of Pharmacy, University of Connecticut, Storrs, CT, USA

Govind Chate Maharashtra Institute of Pharmacy, MIT Campus, Pune, India

Nabo Kumar Chaudhury Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, New Delhi, India

Anisha A. D'Souza Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (Elite status), Deemed University, Mumbai, India

Shilpa M. Dawre Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (Elite status), Deemed University, Mumbai, India

Padma V. Devarajan Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai, India

Fahima Dilnawaz Laboratory of Nanomedicine, Institute of Life Sciences, Chandrasekharpur, Odisha, India

Rinku Dutta Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (Elite status), Deemed University, Mumbai, India

Elias Fattal Faculty of Pharmacy, Institut Galien, University of Paris-Sud, Châtenay-Malabry, France

Chandraiah Godugu National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, Andhra Pradesh, India

Nadège Grabowski Faculty of Pharmacy, Institut Galien Paris-Sud, University of Paris-Sud, Châtenay-Malabry, France

Prem N. Gupta Formulation and Drug Delivery Division, CSIR-Indian Institute of Integrative Medicine, Jammu, India

Rainer Haag Institut für Chemie und Biochemie, Freie Universität Berlin, Berlin, Germany

Hervé Hillaireau Faculty of Pharmacy, Institut Galien Paris-Sud, University of Paris-Sud, Châtenay-Malabry, France

Keiji Hirota Center for Drug Delivery Research, Faculty of Pharmaceutical Sciences, and Japan Center for Physical Pharmaceutics, Research Institute for Science and Technology, Tokyo University of Science, Noda, Chiba, Japan

Jouni Hirvonen Division of Pharmaceutical Technology, University of Helsinki, Helsinki, Finland

Arun K. Iyer Department of Pharmaceutical Sciences, Applebaum College of Pharmacy of Pharmacy, Wayne State University, Detroit, MI

Anjali Jain Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, India

Sanyog Jain Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Ankitkumar S. Jain Department of Pharmaceutics, Bombay College of Pharmacy, Mumbai, India

Vishvesh M. Joshi Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (Elite status), Deemed University, Mumbai, India

Mamta Kapoor Department of Pharmaceutics, University of Minnesota, Minneapolis, MN, USA

Wahid Khan Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, India

Jayant Khandare Maharashtra Institute of Pharmacy, MIT Campus, Pune, India

Varun Kushwah Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Vinod Labhasetwar Department of Biomedical Engineering, Lerner Research Institute and Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH, USA

Kimiko Makino Center for Drug Delivery Research, Faculty of Pharmaceutical Sciences, and Japan Center for Physical Pharmaceutics, Research Institute for Science and Technology, Tokyo University of Science, Noda, Chiba, Japan

Guru Prasad Mohanta Department of Pharmacy, Annamalai University, Annamalai Nagar, Tamil Nadu, India

Anjan Kumar Mohanty Department of Pharmacy, Annamalai University, Annamalai Nagar, Tamil Nadu, India

Eameema Muntimadugu Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, India

Rayasa S. Ramachandra Murthy Faculty of Technology and Engineering, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India

Sumukha Green Ville, Sarvabhauma Nagara, Bilekahalli, Bangalore, India

Mangal S. Nagarsenker Department of Pharmaceutics, Bombay College of Pharmacy, Mumbai, India

Lalit Mohan Negi Department of Pharmaceutics, Jamia Hamdard, New Delhi, India

Giorgia Pastorin Department of Pharmacy, National University of Singapore, Singapore, Singapore

Bhushan S. Pattni Department of Pharmaceutical Sciences, Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA, USA

Chiranjeevi Peetla Department of Biomedical Engineering, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

Leena Peltonen Division of Pharmaceutical Technology, University of Helsinki, Helsinki, Finland

Ramarao Poduri Centre for Chemical and Pharmaceutical Sciences, Central University of Punjab, Bathinda, Punjab, India

D'Arcy Prendergast Department of Pharmacy, National University of Singapore, Singapore, Singapore

Sudha Rana Division of CBRN Defence, Institute of Nuclear Medicine and Allied Sciences, New Delhi, India

Sanjeeb Kumar Sahoo Laboratory of Nanomedicine, Institute of Life Sciences, Chandrasekharpur, Odisha, India

Hélder A. Santos Division of Pharmaceutical Technology, University of Helsinki, Helsinki, Finland

Sanket M. Shah Department of Pharmaceutics, Bombay College of Pharmacy, Mumbai, India

Lipa Shah Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Boston, MA, USA

Rakesh Kumar Sharma Division of CBRN Defence, Institute of Nuclear Medicine and Allied Sciences, New Delhi, India

Rajeev Sharma Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. H.S. Gour Vishwavidyalaya, Sagar, Madhya Pradesh, India

Raman Preet Singh Evaluserve SEZ (Gurgaon) Pvt. Ltd., Gurgaon, India

Sushama Talegaonkar Department of Pharmaceutics, Jamia Hamdard, New Delhi, India

Meghna Talekar Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Boston, MA, USA

Hiroshi Terada Center for Drug Delivery Research, Faculty of Pharmaceutical Sciences, and Japan Center for Physical Pharmaceutics, Research Institute for Science and Technology, Tokyo University of Science, Noda, Chiba, Japan

Niigata University of Pharmacy and Applied Life Sciences, Niigata City, Japan

Vivek S. Thakare Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Kaushik Thanki Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Kiran Todkar Actorius Innovations and Research (AIR), Pune, India

Keishiro Tomoda Center for Drug Delivery Research, Faculty of Pharmaceutical Sciences, and Japan Center for Physical Pharmaceutics, Research Institute for Science and Technology, Tokyo University of Science, Noda, Chiba, Japan

Vladimir P. Torchilin Department of Pharmaceutical Sciences, Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA, USA

Amit Tyagi Division of Nuclear Medicine, Institute of Nuclear Medicine and Allied Sciences, New Delhi, India

Dileep Urimi Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Juliette Vergnaud Faculty of Pharmacy, Institut Galien Paris-Sud, University of Paris-Sud, Châtenay-Malabry, France

Suresh P. Vyas Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. H.S. Gour Vishwavidyalaya, Sagar, Madhya Pradesh, India

About the Editors

Prof. Padma V. Devarajan is Professor in Pharmacy and Head of the Department of Pharmaceutical Sciences and Technology at the Institute of Chemical Technology (ICT), Mumbai, India. She obtained her PhD (Tech) in Pharmaceutics from the Department of Chemical Technology, University of Mumbai, India. Her research interests include nanocarriers for targeted delivery in cancer and infectious diseases, scale-up of nano drug delivery systems (DDS), bioenhancement strategies, and mucosal DDS as alternatives to parenteral administration for protein and nucleic acid delivery. Prof. Devarajan has supervised more than 100 master's and doctoral candidates. Her research is funded by the Government of India and the national and international pharmaceutical industry. She is an author of several book chapters and has publications in peer-reviewed high-impact journals. She has a number of granted patents, with four licensed to the industry. Prof. Devarajan has served as board member, member on the Board of Scientific Advisors, and Chair of the Young Scientist Mentor Protégé Sub-committee of the Controlled Release Society. She is a recipient of the American Association of Indian Pharmaceutical Scientists (AAiPS) Distinguished Educator and Researcher Award, the VASVIK award for Industrial Research to Women, and the Association of Pharmaceutical Teachers of India–C.J. Shishoo Award for Research in Pharmaceutical Sciences, and she is a nominated Fellow of the Maharashtra Academy of Sciences, India.

Dr. Sanyog Jain is currently Associate Professor at the Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Mohali (Punjab), India. He obtained his Ph.D. in Pharmaceutics from Dr. Hari Singh Gour University, Sagar (MP), India. His key research interests include exploring nano drug delivery systems for various biomedical applications including targeted anticancer drug delivery, oral delivery of vaccines, peptides and therapeutics, topical and transdermal drug delivery, gene delivery, and carbon nanotubes. He has patented several nanotechnology-based drug delivery platforms and licensed Tamoxifen-SEDDS Technology for improved breast cancer therapy to industry for commercialization. He has authored several book chapters

and published research papers in high-impact journals. Dr. Jain has won several prestigious awards including Bharat Jyoti (The Glory of India) Award, Alkyl Amine Young Scientist Award, Indian National Science Academy (INSA) Medal for Young Scientists, OPPI Young Scientist Award, and Punjab State Young Scientist Award for outstanding research in the field of drug delivery systems. He has supervised more than 50 master's and doctoral students and a post doc. His research is well funded by various government agencies and pharmaceutical industries. Jain is a widely travelled scientist and has delivered invited talks at numerous prestigious platforms.

Part I
Targeted Drug Delivery: Basic Concepts and Advances

Chapter 1
Targeted Drug Delivery Systems: Strategies and Challenges

Bhushan S. Pattni and Vladimir P. Torchilin

Abbreviations

DDS	Drug delivery system
TDDS	Targeted drug delivery system
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
BBB	Blood–brain barrier
RES	Reticuloendothelial system
PEG	Poly(ethylene) glycol
MNP	Magnetic nanoparticles
SPION	Superparamagnetic iron oxide nanoparticles
ADEPT	Antibody-directed enzyme prodrug therapy
GDEPT	Gene-directed enzyme prodrug therapy
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
HSV	Herpes simplex virus
EPR	Enhanced permeability and retention
SLN	Solid lipid nanoparticle
FDA	Food and Drug Administration
siRNA	Small inhibiting RNA
RNAi	RNA interference
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

B.S. Pattni • V.P. Torchilin (✉)
Department of Pharmaceutical Sciences, Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, 140 The Fenway, Room 211/214, 360 Huntington Avenue, Boston, MA 02115, USA
e-mail: bhushanpharma@gmail.com; v.torchilin@neu.edu

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_1

TB	Tuberculosis
MSNP	Mesoporous silica nanoparticles
PEI	Polyethyleneimine
CNS	Central nervous system
RBC	Red blood cells
PLA	Poly (D,L-lactide)
HAART	Highly active antiretroviral therapy
MDR	Multidrug resistance
AZT	Azidothymidine
BCSFB	Blood–cerebrospinal fluid barrier
CMT	Carrier-mediated transport
RME	Receptor-mediated endocytosis
AME	Absorptive-mediated endocytosis
APO E	Apolipoprotein E
LDL	Low density lipoprotein
CSSS	Cyanoacrylate skin surface stripping
PLGA	Poly (DL-lactide-co-glycolide)
BRB	Blood–retinal barrier
RPE	Retinal pigmented epithelium
P-gp	P-glycoprotein
PepT	Peptide transporters
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
CDDS	Colon targeted drug delivery systems
GIT	Gastrointestinal tract
RISC	RNA-induced silencing complex
CPPs	Cell penetrating peptides

1.1 Introduction

In this complex and ever-evolving world of medicine it has become increasingly important to address the issues of the drug development involving the delivery of specific drugs to their site of action in therapeutically acceptable doses. With the advancement of the pharmaceutical sciences, the industry has certainly observed discovery of several new drug molecules ranging from small molecule drugs to macromolecules like proteins and peptides; but the ultimate goal of achieving disease-free conditions in the patients is often left hanging due to several hurdles relating to physicochemical and molecular intricacies of the "free" drugs and unapproachability and under-dosing of most of the biological/pathological targets. To improve on these situations drug delivery systems (DDS) are employed which could either be a formulation or a device that facilitate the administration of a drug to the body whilst improving its pharmacokinetic and biodistribution profiles and the efficacy and safety of the whole treatment. Targeting the drugs (and DDS) involves the improvement of the specificity of the system towards the pharmacologically relevant target in

the body. Targeted drug delivery systems (TDDS) involve the administration of the DDS to the patient, delivery of the DDS at the target (pathological) site, release of the active ingredients in/around the target, and avoiding nonspecific toxicity in normal cells. The above concept of targeted drugs—magic bullet—was first conceived by Paul Ehrlich in early twentieth century and over the past decades several strategies have developed to achieve targeting [1, 2].

A TDDS can be broadly understood as a system that carries out the following functions:

- Facilitate the therapeutic substance to reach the site of action from the site of administration where the target site can be organ, tissue, cell or even specific cell organelles.
- Release the therapeutic payload in its active form in/around the target site presenting effective therapeutic levels at the site of action.
- Protect the drug/gene from the detrimental effects of environmental factors such as pH, enzymes, etc.
- Avoid toxicity or adverse reactions of the drug/gene on nonspecific normal cells and facilitating administration of lower doses to achieve therapeutic/diagnostic benefits.

Research in the field of targeted drug delivery has given several options of carrying out the above functions:

- Direct targeting to site of action, e.g., topical applications for skin diseases.
- Use of external stimuli, e.g., ultrasound.
- Chemical modification of the drug to make its physicochemical properties ideal for the delivery which includes prodrug approach of attaching a promoiety to the drug.
- Use of nanocarriers like liposomes, polymeric micelles, polymeric nanoparticles, solid lipid nanoparticles which can be functionalized further with attachment of targeting ligands, antibodies.

An efficient TDDS ideally should possess the following properties:

- The drug-conjugate/drug-carrier should reach the intended site of action (organ/tissue/cell/cell organelles) with minimal nonspecific accumulations.
- The chemical conjugation or physical encapsulation of the drug/gene with the targeting ligands or carriers should not inactivate or alter the drug/gene action on the intended site of action; the TDDS should be able to protect the drug from environmental factors such as enzymatic degradation till they reach the target.
- The chemical conjugation or physical encapsulation of the drug/gene with the targeting ligands or carriers should not inactivate or alter the ligand or carrier activity and function to reach the intended site of action.

This chapter has been divided into sections which cover the general strategies of developing TDDS, the use of TDDS in diseases like cancer, HIV/AIDS, tuberculosis, and the use of TDDS to target specific organs and locations. While the targeted drug delivery systems require in-depth study on their own, the intentions of this chapter remain to provide only an overview of the relevant challenges and strategies.

1.2 Targeted Drug Delivery: General Concepts

Targeted drug delivery at the site of action can be carried out by direct techniques usually involving invasiveness: direct injection, catheter [3, 4], gene-gun [5], etc. Though these systems show direct delivery, invasiveness is not patient convenient and expensive to carry out in many cases. As a result, efforts are put into developing TDDS which involve chemical, physical and biological modifications with or without the use of carriers.

Changes done to improve targeting the drug include study of structure activity relationships to improve the physicochemical properties for targeting. Small-molecule drugs intended for brain delivery unable to penetrate the blood–brain barrier (BBB) may be made more lipophilic to aid penetration through the BBB, provided they have small size. Prodrugs can be made to improve the pharmacokinetics of the drugs. Small molecule drugs are chemically modified by attaching "promoeities" rendered pharmacologically inactive and are metabolically activated in vivo into active drugs only after reaching their intended target [6]. Drugs may be conjugated with antibodies, peptides, aptamers, folic acid, etc. to generate targeted prodrugs [7].

On the other hand, the drugs can be incorporated into nanocarriers or nanosystems. These include drug carrier systems like liposomes, polymeric micelles, polymeric nanoparticles, polymer–drug conjugates, nanogels, carbon nanotubes, etc. [8]. The nanosystems are a very efficient way to deliver the drugs or genes. The major advantage of using such systems is that the pharmacokinetic behavior of the drug-loaded nanocarriers depends on the nanosystems rather than the drugs or genes, which makes it easy to control with the help of further targeting. The nanoparticles described in this chapter are <300 nm, unless noted otherwise.

Such drugs/drug carrier systems depend on a few modes of targeting which are broadly classified into passive and active targeting.

1.2.1 Passive (Physiology-Based) Targeting

Passive targeting is present naturally in the human body. Hormones, neurotransmitters, growth factors, etc. have a natural tendency to go and target the receptors at their sites of action, e.g., insulin and insulin receptors. This concept can be applied to the drugs too. The accrual of drugs/drug-carrier systems at the intended site of action by the action of physicochemical and physiological factors is passive targeting [9].

Certain tissues under diseased conditions present opportunities in terms of modified physiologies which can be exploited by passively targeting nanocarriers. A presence of leaky vasculature with large gaps in the blood vessel's epithelial layers has been observed in cases of inflamed tissues in inflammatory bowel disease and inflammatory rheumatoid arthritis [10] and in tumor tissues [11] which make it possible to passively target the administered nanocarriers of appropriate sizes to extravasate into the target tissue. Although the tumor tissue has limited lymphatic drainage [12] and the inflammatory tissues have a functioning lymphatic drainage,

the passive targeting can still benefit the inflammatory diseases. Accumulation of nanocarriers is also observed in the liver due to large fenestrations in it and this can be used for liver targeting in liver diseases. This phenomenon wherein the nanocarriers accumulate into the diseased tissues because of loose fenestrations and/or poorly formed lymphatic drainage is termed as enhanced permeability and retention (EPR) effect [11, 13].

The nanocarriers are largely affected to clearance by the reticulo-endothelial system (RES) comprising of macrophages and mononuclear phagocytes. This fact can be used to passively target the macrophages and even lymph nodes and spleen to treat infections that affect the RES (e.g., leishmaniasis and malaria) [14].

Often modifications (e.g., attachment of polyethylene glycol; PEG) are made on nanocarriers to make them long-circulating, avoiding the RES and granting them time to accumulate at target sites in high amounts (long-circulating nanocarriers) [15].

Passive targeting also benefits from the presence of internal stimuli, such as pH difference (e.g., low pH in tumor microenvironment [16]), redox systems (e.g., exploiting high glutathione in cancer [17]), etc. in the diseased tissues. Stimuli-sensitive drug targeting systems will be spurred by such stimuli to release the drug only at the target site and spare the normal tissues. Such stimuli-responsive systems have been extensively studied [18–22].

1.2.2 Active Targeting

While significant results have been observed with passive targeting, the pursuit of better control on accurate drug delivery has led to a lot of research in active targeting methods. Appropriate modifications and functionalization on the drugs or drug carriers afford them affinity towards specific receptors/markers on cells, tissues or organs. Factors such as the disease, the intended target organ, and a larger presence of targetable components on the target organ/cell (e.g., transferrin receptors in tumor) than in normal cells are taken into consideration while deciding on the targeting moiety to be attached to the therapeutic substances. Modifications on the drugs or drug carriers can involve the use of ligands such as peptides, antibodies, sugars, lectins, etc. Thus, on administration to the body, the targeting moieties will enable the drug/drug-carriers to efficiently reach only the intended sites of action and avoid nonspecific accumulations and related side effects.

Apart from the administration of such actively targeted systems, there are technologies available to further control the delivery system which are covered below.

1.2.2.1 Targeting Mediated by External Stimuli

External stimulus, such as magnetic fields and ultrasound, acting on on nanocarriers, are employed to perform imaging, to target and release drugs from the nanocarriers at the intended site of action. The benefits of using this mode of active

targeting are: real-time targeting, targeting deep-seated tissues, simultaneous imaging and therapy. The approach can also combine different external stimuli, for example, ultrasound and magnetic field, for enhanced targeting and efficiency.

The use of magnetic nanoparticles (MNP) as imaging agents for magnetic resonance imaging, magnetic drug targeting and hyperthermia treatment is well explored in the field of targeted drug delivery. The MNP can be either metallic or bimetallic or superparamagnetic iron oxide nanoparticles (SPION); among them SPION are widely studied for biomedical applications because of nontoxic nature, ability to be functionalized with different targeting coatings and can encapsulate drugs in reasonable quantity. Optimizing the MNP as well as the external magnet is of prime importance because on application of magnetic field, they must be able to generate enough magnetic moments and magnetic gradient that the MNP can overcome the force of the blood-flow (rating from 0.05–50 cm/s) depending on the target area [23, 24]. The MNP have found several uses in thrombolytic therapy [25, 26], intravascular imaging and cardiovascular diseases [27–29], tumor imaging and treatment [30–35], as well as delivery across the blood–brain barrier [36, 37].

Ultrasound has been used previously for contrast imaging, and it is explored at length for use in drug delivery. Ultrasound mediated targeting can lead to disruption of the drug-loaded carriers (microbubbles, micelles, etc.) causing drug release; exact mechanism of release is still under study. Furthermore, ultrasound focusing has also been found to cause reversible disruptions (Fig. 1.1) in the intravascular endothelial layers creating pores for the drug to enter the extracellular space of the target tissue. This occurrence was also observed with blood–brain barrier/blood–tumor

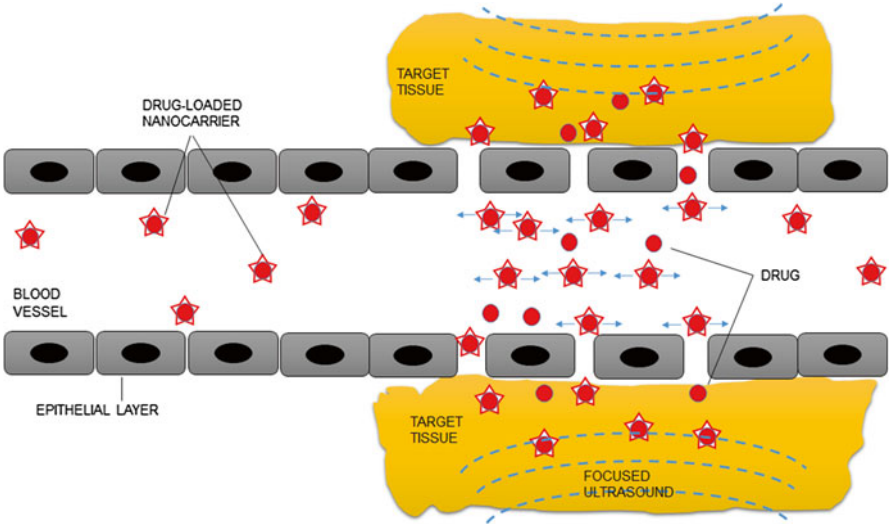

Fig. 1.1 External stimulus of focused ultrasound leads to (**a**) reversible disruptions and gaps in the epithelial cell layer allowing drugs/drug nanocarriers to escape the blood vessels into the target tissues, (**b**) disruptions to the nanocarriers to release the drugs around the target tissue

barrier [38–40]. Another benefit of using the ultrasound-mediated delivery is in targeted hyperthermia when combined with temperature-sensitive nanocarriers [41]. The generation of hyperthermia can prove cytotoxic for nearby tissues and in case of tumor treatment this is highly beneficial; the hyperthermia will kill tumor periphery cells and open the way for simultaneously administered drugs to enter the core of tumor tissue for enhanced killing. Studies with ultrasound therapy find applications in tumor imaging [42], tumor treatment [43–48], thrombosis [49, 50], cardiovascular diseases [51]. This mode of targeting also gives the opportunity to image and trigger release of the drug at the same time [52–54], and also to combine it with magnetic field applications for enhanced benefits [55, 56].

1.2.2.2 Antibody-Directed Enzyme Prodrug Therapy (ADEPT)

This approach is a two-step approach: (1) an activating enzyme is specifically delivered to intended site of action with a targeting antibody (e.g., tumor-specific antibody, anti-TAG-72) (2) a subsequent administration of substrate prodrug. The advantage with such a system is a single enzyme at the target site can activate multiple prodrugs and increase the load at the target site. A 3-phase ADEPT was performed in human ovarian carcinoma xenografts in mice. First, an enzyme–antibody complex—AB57-F(ab′)$_2$-CPG2—was allowed to localize in the tumor followed by a wash step of removing blood CPG2 by anti-CPG2 antibody administration to avoid nonspecific activation of the prodrug. Lastly, a benzoic acid mustard-derived prodrug was injected leading to tumor growth delay [57].

A modification of this approach is the *gene-directed enzyme prodrug therapy* (GDEPT). Here, instead of antibody-targeted enzyme, a gene is targeted to the intended site where it transcribes and translates to produce enzyme intracellularly which acts on the subsequently administered prodrug [7].

1.2.2.3 Targeting in Gene Therapy

This section primarily discusses about the strategies required to transport genes into their required site of action which is either the cytoplasm or the nucleus. Gene gun as mentioned previously is a physical method to directly transfer DNA and RNA with high transfection efficiency but because it is invasive and requires special setup it is not widely preferred. The current gene therapy employs viral and nonviral vectors.

The viruses have a unique ability to transfer their genes into cells. This function can be utilized to deliver genes. The bottom line, though, is that the viral vectors have to be modified to be devoid of virulent pathogenesis and replicative genes. The commonly used viral vectors include adenovirus, baculovirus, herpes simplex virus type 1 (HSV-1), etc. [58–60]. Utmost care has to be taken when designing these systems because the viral vectors are notorious for adverse effects such as inflammatory and immune responses, activation of latent infections, incorporation of transgenes into the host genomes and permanent expression persistence.

Nonviral methods to target gene transfer are not proficient with transfection efficiency but they are generally safer than the viral vectors. Polymeric micelles, liposomes and other nanocarriers have been studied to deliver the nucleic acids [61–63].

Thus presented are the classifications and the strategies of targeted drug/gene delivery systems. The next few sections feature the benefits of TDDS in various diseases.

1.3 Targeted Drug Delivery: Disease-Based Strategies

1.3.1 Cancer Specific Strategies

Cancer is uncontrolled growth of abnormal cells characterized by mutations which help the cells to proliferate, avoid apoptosis and develop survival proteins. Perhaps, cancer is the disease on which the most research in targeted drug delivery is focused and for a good reason. Even though chemotherapy, surgery and radiotherapy are available, the cancer manages to remit and regrow in most of the cases. It is a disease where the survival of patients is measured in weeks and months only even if some of the most advanced drugs are used. There are several reasons why cancer is so difficult to treat.

To begin with, the tumor microenvironment (higher interstitial fluid pressure, low extracellular pH, and formation of irregular tumor vasculature) as well as the cellular level (over-expression of efflux transporters, defective apoptotic machineries, and altered molecular targets) attribute multidrug resistance (MDR) towards the drugs [64]. Second, most of the chemotherapeutic drugs do not possess desirable physicochemical and pharmacokinetic properties. They have low solubility and stability, are highly nonspecific in nature and show high toxicities and are inconvenient to the patients. Therefore, targeted therapy in cancer plays a very important role. Nanopreparations are primarily employed to achieve this task [15] and approved products include Myocet® (liposomal doxorubicin), Daunoxome® (liposomal daunorubicin), Doxil® (liposomal doxorubicin), Depocyt® (liposomal cytarabine), Oncaspar® (monomethoxypolyethylene glycolol conjugation to L-asparaginase), Genexol-PM® (paclitaxel-loaded polymeric micelle), Abraxane® (albumin-bound paclitaxel particles), etc. Apart from these there are several other studies in various stages of clinical trials. Antibodies directed towards cancer therapy include Rituxan® (rituximab), Herceptin® (trastuzumab), Campath® (alemtuzumab), etc.

Methods other than using nanopreparations have also been examined. A recent study used chimeric antigen receptor-modified T cells targeted towards the CD19 expressing chronic lymphocytic leukemia (CLL). This research was performed in two children with complete remission of cancer with one having a relapse. The researchers have called for further work but the underlined principle exhibits the benefits of lentiviral-vector targeting [65].

The nanocarriers can target the tumor via passive targeting and active targeting. As explained previously, passive targeting involves the physicochemical properties of nanocarriers and the physiological factors. The tumors are rapidly proliferating

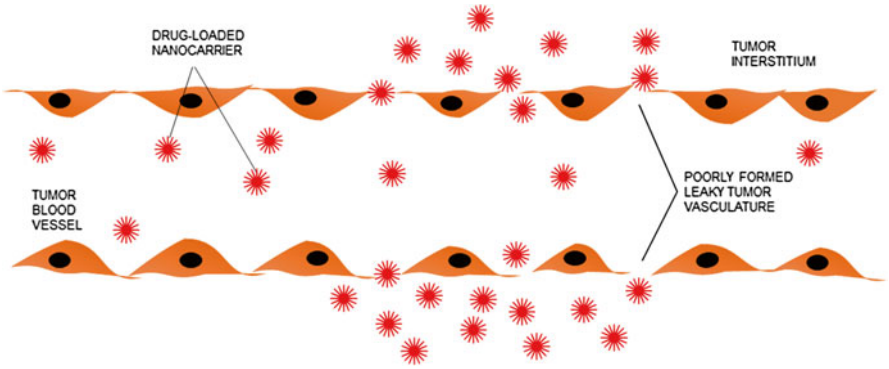

Fig. 1.2 Enhanced Permeability and Retention (EPR)—Poorly formed tumor vasculature with leaky vessels and meager lymphatic drainage can be exploited by passively targeting the nanocarriers up to size of 500 nm

cells and they require nutrients in large amounts. As a result, the tumors expedite the blood vessel formation leading to irregular and leaky tumor vasculature. The gaps in the endothelial layers allow the nanoparticles up to 500 nm in size to extravasate into the tumor tissue (Fig. 1.2). The absence of a proper lymphatic drainage helps to accumulate the drug-loaded nanoparticles in the tumor tissue. EPR is responsible for passive targeting in tumors [13, 66]. To enhance this effect the nanopreparations are modified by motifs like polyethylene glycol (PEG). In effect the presence of PEG modifications help nanocarriers avoid RES uptake and they circulate longer in the blood allowing them more time to accumulate in the tumor tissue.

Active targeting of the nanopreparations involve modifications that will not only help target the tumor tissue but also overcome the resistance factors. For instance, ATP-binding cassette transporters like P-glycoprotein on the cell membrane are responsible for efflux of several drugs and generating multidrug resistance [64]. To bypass such efflux transporters, drugs have been administered in nanocarriers with improved tumor inhibition efficiencies [67, 68]. Nanocarriers can also be actively targeted by conjugation with targeting ligands such as transferrin, folate, antibodies, etc. [69–73]. Interesting strategies to target tumor also involve stimuli-responsive nanoparticles which may even be dual targeted to facilitate step-by-step entry into the tumor cells [18, 74–76]. Several other examples of targeting various facets of cancer are discussed in these reviews [64, 77, 78]. We are not discussing a huge area of tumor targeting in details, since a broad variety of recent publications exist on this subject [79–83].

1.3.2 Targeted Drug Delivery Towards Infections

Infections by bacteria, fungi, virus and other microorganisms were the cause of widespread diseases and fatalities throughout the world in the early part of the twentieth century. As the understanding of the pathology expanded and the

discovery of antibacterial penicillin came through, the number of antimicrobial and antiviral agents have been developed. Essentially the antimicrobial inhibit or interrupt important cell cycle processes of the microorganisms and kill them. Even though we have been able to reduce the huge number of fatalities due to preventive and therapeutic actions, the menace of infections is still present in several developing and underdeveloped nations. Most of such countries are unable to meet the demands of antimicrobials to control infections such as malaria, tuberculosis (TB), HIV/AIDS, cholera and many more. As a result there is still a prevalence of such diseases. Moreover, it has now been observed that the unchecked abuse of antibacterial agents have resulted in developing resistant strains of the once sensitive pathogens [84]. While new antibiotics (oritavancin, telavancin, etc.) are in the various stages of drug discovery and clinical trials, their development will take time to reach the market [85, 86]. This situation demands for TDDS for the current class of drugs.

A lot of interest has been generated to develop TDDS especially the ones using nanoparticles, for infectious diseases, with their simplest advantages being the ability to deliver a variety of drugs and even genes, the ability to give sustained and targeted delivery while avoiding the toxicity as well as the ability to deliver combination of multiple drugs at the same time to overcome drug resistance. Liposomes, SLN, polymeric nanoparticles and other forms of nanoparticles are frequently studied for the TDDS development [87]. An FDA approved injectable liposomal formulation for amphotericin B, AmBisome (Gilead Sciences, Foster City, California, USA) is employed for the use against several infections. A targeted drug delivery for the AmBisome was performed in mice models of disseminated aspergillosis, and it was observed that the liposomal formulation was superior in efficacy compared to the amphotericin B deoxycholate in sterile water [88].

Techniques similar to observed in targeted cancer treatment, ligands, antibodies, peptides, etc. can be attached to the nanocarriers to enhance the efficiency of the anti-infectious treatments. The cell walls of *Helicobacter pylori* have carbohydrate receptors to which lectin-conjugated nanoparticles could specifically bind. Thus, a targeted preparation of lectin-conjugated gliadin nanoparticles was studied for receptor-mediated targeting towards *H. pylori* [89]. Studies have also been undertaken to deliver genes to infected cells. A TDDS for dengue virus infection was recently developed to deliver siRNA in a novel manner. The Dengue virus is known to affect the human dendritic cells as well as macrophages. Hence, a dendritic cell-targeting 12-mer peptide (DC3) was fused with nona-D-arginine (9dR) residues to form a DC3-9dR-mediated delivery of siRNA targeting the tumor necrosis factor alpha (TNF-α) which was able to target the dendritic cells as well as deliver the siRNA effectively; the result of which was suppressed virus replication and virus-related symptoms [90].

The remaining part of this section is an overview of TDDS developed for specific infections.

1.3.2.1 Tuberculosis (TB)

According to World health Organization (WHO), *Mycobacterium tuberculosis*, the bacteria responsible for tuberculosis, has caused infection in 8.6 million people worldwide by 2012. The infection is transmitted through air and it deposits in the alveolar region of the lungs. Current therapy employs antibiotics like rifampicin, isoniazid, ethionamide, etc. and often combination therapy is prescribed to attack the *M. tuberculosis*. Still, the bacteria has developed resistance against most of the drugs and when the condition is aggravated by concomitant HIV/AIDS presence, patients usually do not survive the infection well [91, 92]. Also, the drugs present serious side effects such as hepatotoxicity [93].

Hence, research is undertaken to develop targeted drug delivery for TB. Since the bacteria are taken up by the macrophages/monocytes in the lungs, the opportunity for targeting receptors such as lectin (mannose) receptors, immunoglobulin receptors, complement receptors, etc. expressed on such alveolar macrophages is presented [94]. Moreover, the macrophages have an innate response of phagocytizing the nanoparticles, hence targeting them makes a valid choice for treatment of TB. Most of the TDDS developed for TB prefer the pulmonary route of administration because the TB infection is primarily localized in the lungs.

Active targeting of the lectin receptor on the alveolar macrophages of rats via pulmonary administration of mannose-coated liposomes containing ciprofloxacin was performed giving increased drug uptake in the lungs as compared to the free drug; the plasma concentration of ciprofloxacin was also low with the targeted liposomes in comparison to the free drug exhibiting the benefits of using the targeting strategy [95]. A similar study was carried out via intratracheal administration of different concentrations of mannose in mannose-coated liposomes resulting in selective targeting and increased uptake in the alveolar macrophages of the rats [96]. Pandey and Khuller developed nebulized solid lipid particles incorporating a combination of rifampicin, isoniazid, and pyrazinamide for bronchoalveolar drug delivery in *M. tuberculosis* infected guinea pigs. As compared to 46 daily doses of orally administered drugs, the nebulized formulation achieved a complete removal of the tubercle bacilli from lungs and spleen after just seven doses of administration, each dose administered periodically on every seventh day. Moreover, hepatotoxicity was not observed suggesting a sound basis for improved drug bioavailability and treatment of tuberculosis using the nebulized SLN formulation [97]. An interesting approach was adopted by Clemens et al. to target the TB-infected macrophages with functionalized mesoporous silica nanoparticles (MSNP). Two formulations were developed—a rifampicin-loaded MSNP coated with polyethyleneimine (PEI) and an isoniazid-loaded MSNP equipped with cyclodextrin-based pH-operated valve. In vitro experiments highlighted that the MSNP were internalized efficiently by the human macrophages and because of their functionalized nature, the MSNP escaped the acidic endosomes delivering the drugs into the cytoplasm. Thus, the functionalized MSNP demonstrated targeted intracellular delivery into the macrophages [93].

1.3.2.2 Malaria

Malaria is also a widely spread disease similar to TB with an estimated 216 million people affected in 2010 [98]. This disease is caused by the four species of the parasitic protozoans of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Transmission of these parasites occurs through the bites of infected female *Anopheles* mosquito. The life cycle of the *Plasmodium* in the human host goes through the red blood cells (RBCs) and the hepatocytes making them the main parasitic targets. Moreover, the *P. falciparum* is involved in infecting the CNS often leading to fatality.

The current chemotherapy focuses on reducing the parasitic load in the infected cells and the choice of the drug is dependent on the *Plasmodium* species involved in infection. Even though chemotherapy is available, often complete treatment falls short. Apart from the factors related to the spread of the disease, drug resistance and the protozoan life cycle itself are responsible for the lack of complete therapy. Also, as with other drugs, side effects such as neuropsychiatric reactions, hypoglycemia, arrhythmia, hepatitis, agranulocytosis, anemia, and even life threatening reactions have been observed [14]. Because of the serious implications of the current therapy in terms of side effects and development of drug resistance, targeted drug delivery is important in the treatment of malaria. The knowledge that the parasites infect the RBCs and the hepatocytes mainly can be exploited by developing TDDS directed specifically towards them.

Chloroquine-resistant transporters develop as resistance mechanisms on the digestive vacuole membranes of the *Plasmodium*, the main site of action of chloroquine. A strategy to develop nanosystems sensitive to the pH difference in the intracellular compartments is suggested to avoid the chloroquine-resistant transporters and increase the drug payload in the infected cells. Chloroquine diphosphate was loaded into pH-sensitive liposomes and the release of the drug was estimated in vitro in simulated physiological pH conditions [14, 99]. Infected hepatocytes were actively targeted with liposomes incorporating peptides from the *Plasmodium* circumsporozoite protein in a series of experiments. It has been demonstrated that the liposomes accumulate rapidly and selectively in adult mouse livers. The targeting mechanism has been elucidated to be the binding of the targeted liposomes to the heparan sulfate proteoglycans in a fashion similar to the development of heparan sulfate immunoreactivity [100–102]. These proof-of-concept studies exhibit the possibility of development of a strategy to target the hepatocytes with antimalarials.

Halofantrine was intravenously injected in *P. berghei* infected mice as a formulation of nanocapsules prepared with either poly (D,L-lactide) (PLA) homopolymer or PEGylated PLA. The PEGylated nanocapsules were observed to be both long-circulating and cytotoxic to the parasites thus exhibiting passive targeting [103]. Considering that free halofantrine is usually involved in causing arrhythmia as a side effect, the nanocapsules would also benefit in avoiding such adverse reactions. In the case of *P. falciparum* infections, the CNS is usually infected. Accordingly, TDDS to overcome the BBB and facilitate passage of antimalarials would be beneficial to the therapy. The transferrin receptors in the BBB were targeted with transferrin-conjugated SLN loaded with quinine dihydrochloride. In vitro and

in vivo examinations showed higher percentage of the drug in the brain as compared to untargeted SLN loaded with the drug as well as free drug [104]. Other strategies that may be employed include antibody-directed targeted liposomes towards infected macrophages [105].

Recent vaccine development efforts have resulted in a new promising vaccine (RTS,S) for the first time for malaria (approval pending) [98, 106, 107]. This may lead to newer paradigms too in the prevention and treatment of malaria.

1.3.2.3 Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS)

As per 2013 statistics provided by WHO, there are more than 35 million people worldwide who are infected with HIV/AIDS and the number keeps increasing. This disease is responsible for affecting the immune system the initial symptoms of which are like influenza and after a dormant period the immune system is severely compromised and in addition the patient is also exposed to opportunistic infections (e.g., TB, malaria) and even tumors.

The current antiretroviral therapy targets the steps along the life cycle of the HIV replication in the host cells. Briefly the steps are shown in Fig. 1.3 and include attachment and fusion of the HIV to host cell surface esp. $CD4^+$ cells, release of the viral core into the cell cytoplasm, reverse transcription of viral RNA into a double stranded DNA, integration into the host chromosome, protein synthesis and translation and followed by budding and release of mature virus into the extracellular region ready to

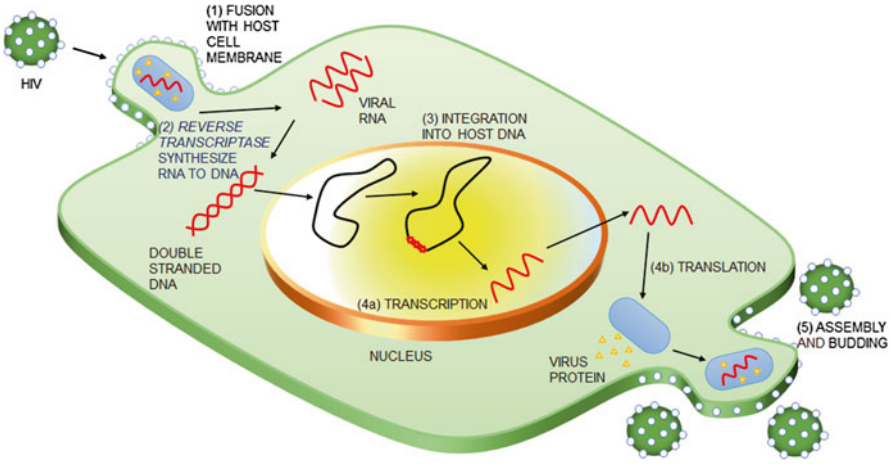

Fig. 1.3 HIV life cycle—(*1*) The first step involves fusion of the HIV with cell membrane of host cells expressing CD4+ and deliver the viral genome in the process. (*2*) Utilizing reverse transcriptase the viral RNA is reverse transcribed into a DNA which enters into the nucleus and (*3*) integrates with the host DNA. The next step leads towards viral protein synthesis (*4a*) and (*4b*) from where the viral proteins are assembled and after budding (*5*) off from the host mature HIV particles are released

affect other cells. The HIV-1 forms cellular reservoirs (dormant $CD4^+$ lymphocytes, macrophage, and dendritic cells) and anatomical reservoirs (CNS, male genitalia) [108, 109]. The current chemotherapy includes nucleoside reverse transcriptase inhibitors, nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, and fusion and integrase inhibitors. Often, a combinatorial therapy is used referred to as highly active antiretroviral therapy (HAART). The use of such a chemotherapy has definitely aided patients in terms of improved survival rates but there are several deficiencies still haunting the HIV/AIDS patients. Frequent dosing, adverse reactions of the drugs, development of MDR, inaccessibility of the anatomical reservoirs are a few of the issues in these therapies.

On the other hand, the cycle of the HIV as well as its reservoirs in the body create an opportunity to develop TDDS against them. For example macrophage can be actively targeted with the help of targeting ligands attached to drug loaded nanocarriers against mannose receptor, formyl peptide receptor 1 and other similar receptors on the macrophages [110]. In such a manner the anatomical reservoir of HIV—the brain can also be targeted. Drug delivery to the brain is a difficult task and strategies for the same are explained in detail in later sections. As an example, water-soluble antiviral drug azidothymidine (AZT) was encapsulated within PEGylated nanoparticles with surface functionalization by transferrin. In vitro and in vivo evaluations confirmed that these TDDS targeted the transferrin receptors in the rat brains and enhanced the brain localization of AZT [111]. Another study was performed to actively target the lymphatic system where the HIV is known to colonize and form reservoirs. Liposomes loaded with zidovudine were surface modified with a lymphatic site-specific ligand—mannose—and compared against negatively and positively charged liposomes as well as unmodified liposomes. It was observed that the surface modified liposomes especially mannose coated were effective in uptake and localization into the lymph nodes and the spleen [112]. This study illustrated the benefits of targeting in improving the drug load in the lymphatic system to eradicate the HIV. Yet another study with poly(ethyleneoxide)-modified poly(epsilon-caprolactone) nanoparticles loaded with radiolabeled [^{3}H]-saquinavir demonstrated significant uptake and prolonged intracellular drug residence by macrophages in in vitro analysis [113]. Similar work of using TDDS in HIV/AIDS has been covered in these reviews [109, 114–116].

Thus, it is clearly observed that targeted drug delivery plays an important role in the therapy for infections.

1.4 Targeted Drug Delivery: Specific Location-Based Strategies

In this section challenges of targeted drug delivery to specific organs and organelles are discussed for which specific targeting strategies need to be employed as each of them presents specific challenges to drug delivery.

1.4.1 Blood–Brain Barrier (BBB) Targeted Delivery

The brain is a very difficult organ to deliver the drugs to because it is very well protected by the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). The BBB is highly specific in allowing transport and has two main functions by which it protects the brain and maintains its homeostasis: (1) supplying the brain with nutrients like glucose and (2) disallow harmful substances to pass through. As depicted in the Fig. 1.4, the BBB comprises an endothelial cell layer with tight intracellular junctions, a basement membrane and feet processes of pericytes and astrocytes. Apart from the tight endothelial junctions which form a physical barrier, the BBB also possesses enzymes and active energy-dependent efflux transporters which respectively inactivate the drug and exude the drug back into the blood from the endothelial cells [117]. The difficulty thus presented results in 98 % of small molecule drugs and almost 100 % of large-molecule drugs to not pass through the BBB to enter the brain. The only small molecule drugs that cross the BBB have high lipid solubility and a low molecular mass of <400–500 Da [118]. Still, the BBB consists of luminal and abluminal membranes which house the transport systems responsible for blood–brain and brain–blood transport of nutrients such as glucose, proteins, and peptides. In case of drug targeting such transport systems can be exploited to specifically target the drug to the brain.

As shown in the Fig. 1.5, different transport mechanisms responsible for transport across the BBB are [119]:

- paracellular transport (non-competitive movement of water soluble compounds through the tight epithelial junctions);
- transcellular transport (non-competitive movement of lipid soluble compounds through the epithelial cells from the luminal side to the abluminal side);

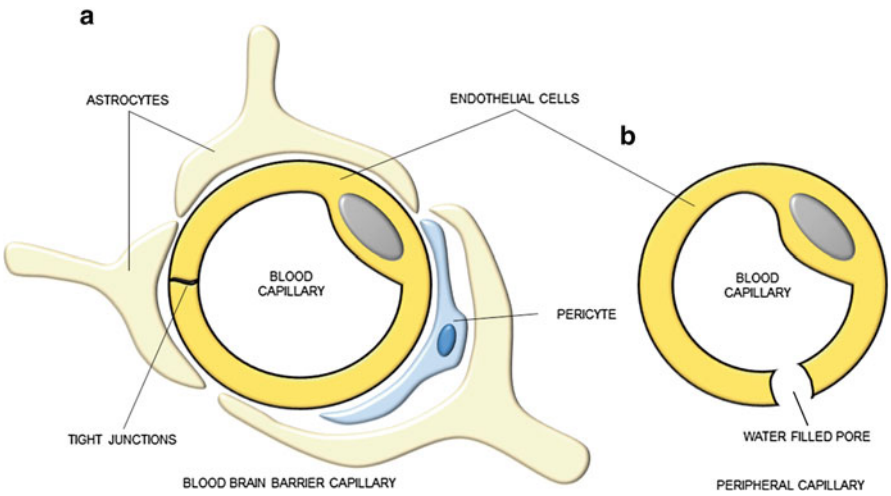

Fig. 1.4 Structure of blood–brain barrier (**a**) in comparison with peripheral capillaries (**b**)

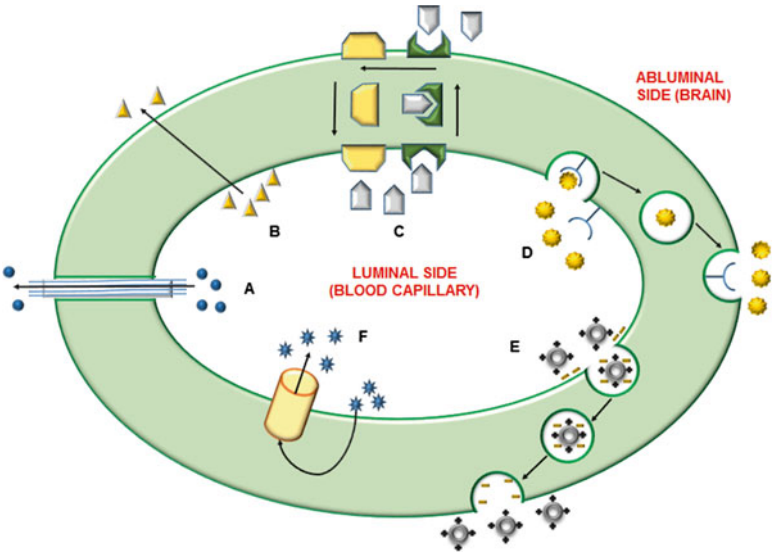

Fig. 1.5 BBB Transport—(**a**) Paracellular transport (water-soluble agents), (**b**) Transcellular pathway (lipid-soluble agents), (**c**) Carrier-mediated endocytosis (*CME* glucose, proteins, etc.), (**d**) Receptor-mediated endocytosis (*RME* transferrin, insulin), (**e**) Absorptive-mediated endocytosis (*AME* cationized agents) and (**f**) Active efflux transporter (drug substrates)

- carrier-mediated transport (CMT) (transport of compounds such as glucose, amino acids, proteins; also includes the efflux transporters on the luminal side like ABC transporters);
- receptor-mediated endocytosis (RME) (receptors for molecules like insulin and transferrin are present which transport the ligands on binding to the receptors);
- absorptive-mediated endocytosis (AME) (transport of plasma proteins like albumin after cationization).

Of course, diseased states like brain tumor, meningitis, infections among others can affect the structure and function of the BBB.

Drugs can be delivered to the brain by direct physical targeting and completely avoiding the BBB. The techniques employed for this include invasive strategies where the drugs are directly injected into the brain after drilling a hole in the head or implants carrying the therapeutics are surgically placed into the brain. Clinical studies were carried out with Gliadel® wafer containing carmustine (chemotherapeutic drug) implants in patients undergoing initial treatment for high-grade malignant glioma and it was observed that in combination with radiation therapy the Gliadel® wafers produced a survival advantage at 2 and 3 years confirming that the drug showed its effect best when it was delivered directly to the cerebral parenchyma [120]. Similar techniques have been used with devices such as Ommaya® reservoir pump, MiniMed PIMS® system, Medtronic SynchroMed® system, and DUROS™, among others [117, 121]. Utilizing the cerebrospinal fluid of the ventricles as a drug

depot via intraventricular injection is another invasive strategy employing the use of a catheter and relying on drug diffusion to various areas of the brain. Apart from delivering the drugs to the brain directly and avoiding the BBB, these methods also avoid the systemic exposure and related toxicity of the drugs as well as requirement of high doses to reach minimum therapeutic dose at the site of action. However, these systems come with their side effects, which include high chances of infections at the site of administration, catheter obstructions, cerebral edemas in case of high concentrations of the drugs in the parenchyma and massive patient discomfort in most cases. Moreover, there is the imminent cost of surgeries required for these delicate procedures and they would be required periodically when the implanted drug reservoirs get empty.

Non-physical targeting of the BBB and the brain which involves chemistry and biology based approaches, thus avoiding the surgical procedures, are also employed. One such technique relies on the temporary disruption of the BBB and increased passage of the concurrently administered therapeutic agents [117, 121, 122]. Administration of hypertonic/hyperosmotic solutions can cause shrinking of the endothelial cells and opening of the tight endothelial junctions due to osmotic pressure differences and the coadministered drugs can thus pass through. A commonly employed intracarotid delivery of a hypertonic solution of mannitol disrupts the BBB transiently and allows for the passage of the drugs [123–126]. BBB disruption has also been reported with the use of the mediators of the inflammatory response (leukotrienes, vasoactive peptides), bradykinin and alkylglycerols [127, 128]. In a way, such strategies are also invasive, although not physically, because they disrupt the natural BBB. They have showed potential in improved drug delivery but they also bring in the chances of exposure of the brain to the infectious agents and toxins and the possibilities of neuropathological changes like infarction, learning disabilities among others [122, 129].

Chemical modifications such as increasing lipophilicity of the drug can be exploited to deliver them via diffusion across the BBB. Of course, the drug still has to be of small molecular mass otherwise it will not cross the BBB. An example is the increased BBB delivery of highly lipid form of morphine: diacetylmorphine/heroin which is a prodrug form of morphine [130]. Nanocarriers such as solid lipid nanoparticles (SLN) or polymeric nanoparticles can also be used to improve the diffusion of the drugs across the BBB. Studies carried out by Yang et al. showed improved concentrations of camptothecin in the mouse brain when administered in the SLN as compared to the solution form of the drug. It was postulated that the improved concentrations were due to endocytosis and simple diffusion across the BBB [131].

The most promising BBB/brain targeted drug delivery is via the active targeting of the transporter mechanisms, namely the CMT, AME and the RME, and avoiding the efflux transporters on the BBB. The CMT in the BBB involves transporters/carriers such as GLUT1 (for glucose), LAT1 (for large neutral amino acids), CNT2 (for adenosine), MCT1 (for lactate) responsible for the BBB crossing of the respective nutrients [132]. Perhaps, the best example of CMT targeting is the use of L-DOPA, a prodrug of dopamine, in patients of Parkinson's disease which targets the LAT1 transporters and efficiently crosses the BBB [133]. After crossing the BBB,

the L-DOPA is converted to the active dopamine by decarboxylases in the abluminal side of the BBB effectively locking dopamine in the brain. Other examples include the use of melphalan (for brain cancer) and gabapentin (γ-amino acid) recognized by the LAT1 transporter.

A method of targeted delivery for large molecular drugs, such as proteins and peptides has been suggested. It involves cationization of the molecules or conjugation of the molecules with cationized albumin or cationized antibodies, thus forming chimeric peptides [134]. This process employs electrostatic interactions of the cationized drugs with the anionic charges on the luminal side of the BBB and the brain facilitating the AME transport of the drugs [135–137].

The BBB consists of receptors such as insulin receptor or transferrin receptor for the endogenous insulin, or transferrin, to transport the latter to the brain via RME. The expression of these receptors on the BBB is more than that in the normal cells, thus representing an opportunity to conjugate the therapeutic drugs or nanocarriers with targeting ligands or peptidomimetic antibodies and gain access to the BBB transport. Anti-transferrin receptor OX26 monoclonal antibody (mAB) has been the subject of several studies to conjugate the drugs as well as genes and target the transferrin receptor [138–141]. Humanized insulin receptor mAB was used by Pardridge et al. to demonstrate transport across the BBB in the Rhesus monkey and it can be used to target the insulin receptors [142, 143]. However, transferrin is present in high amounts endogenously which can compete with the targeted therapeutics while the insulin receptor targeting can also result in nonspecific effects in the body periphery [142]. Another well studied receptor for the RME across the BBB is the low density lipoprotein receptor (LDL). Surfactants such as polysorbate 80 have been attached to several nanocarriers to improve the BBB targeting and transport. When administered intravenously such surfactant-attached nanocarriers interact with plasma proteins like apolipoprotein E (APO E) which is recognized by the LDL receptors, and the targeted delivery is achieved [144–151]. Avidin/Biotin strategy as well as ADEPT has also been employed for targeted brain delivery [117, 152].

1.4.2 Targeting Drug Delivery to the Skin with Highlight on the Follicular Pathway

While the pharmaceutical market is flooded with thousands of formulations for skin delivery of drugs including free drugs in creams, ointments, lotions, dermal patches or sprays, this section focuses on targeted preparations for skin diseases. While the creams and similar preparations can be applied topically, the question needs to be asked whether the definition of targeted systems applies here. In case of free drug formulations, often the case is that the drug does not penetrate the skin because of the tight stratum corneum. Moreover, skin formulations like creams or lotions tend to wash away lowering the drug presence on the skin. Thus, formulations such as nanopreparations like liposomes, solid lipid nanoparticles, and dendrimers are studied to enhance permeation through skin and target the viable epidermis as well

as create stable drug reservoirs [153, 154]. Patients with acne, skin cancer, psoriasis, or infections can benefit from such preparations delivering drugs such as dithranol, miconazole nitrate, and isotretinoin, among others [155–161].

From past few years the *follicular pathway* has gained importance as a targeted drug delivery site and it is now considered as a subject of its own study apart from the topical/transdermal delivery. Initially, it was thought that the topically applied formulations would penetrate the stratum corneum, it has been seen that more penetration is observed through the hair follicles [154]. Of course, the follicular presentation occurs in a variable amount throughout the body (no hair follicles in the palms, soles of feet and the lips) with the highest follicular density observed in forehead and the sural making them one of the most accessible target sites. Targeting the follicular pathway demands an understanding of the pilosebaceous unit which is the integrated structure of the hair follicle, hair shaft, adjoining arrector pili muscle, and the associated sebaceous gland as shown in Fig. 1.6. The sebaceous glands as well as the bulge region are attractive target areas as the former is involved in diseases like acne, alopecia and anatomically capillary rich while the latter is rich in stem cells in charge of follicle reconstitution. Other targets can be the hair follicle infundibulum, the hair follicle papilla and the hair matrix [162].

It has been suggested that hair follicles in an active (open) state (sebum flow and/or hair growth) are accessible for penetration as compared to the inactive states. To improve the penetration, often pretreatments to remove the cellular debris from the stratum corneum are carried out with cyanoacrylate skin surface stripping (CSSS) [163–165]. Consideration must also be given to the phase of the hair growth cycle, i.e., anagen (growth phase), catagen (end of mitosis, cell death of lower follicle segment), telogen (resting phase), exogen (release of telogen fibers), and kenogen (time between exogen and subsequent anagen) while developing a delivery system [166, 167]. Furthermore, it was observed that systemic delivery through the follicular pathway was possible. Caffeine in shampoo applied topically to the skin

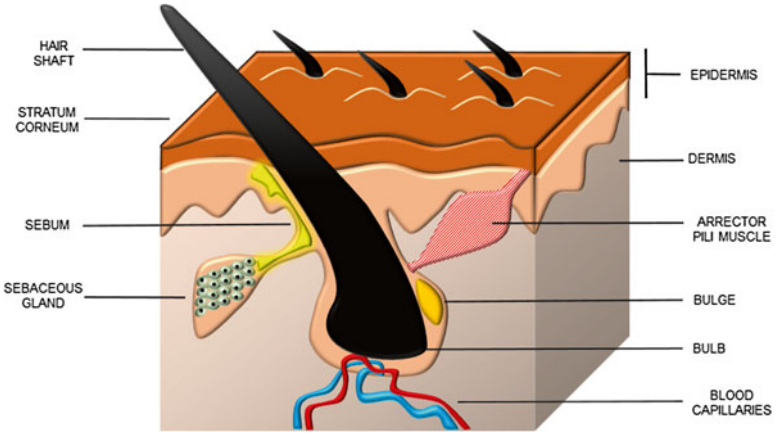

Fig. 1.6 Pilosebaceous unit

appeared in the blood 5 min later due to the presence of blood vessels around the sebaceous gland [168].

Studies exploiting the follicular pathway use liposomes generally because of the favorable characteristics, amphiphilic nature, and high loading capacities [169]. A recent study reported the use of minoxidil-loaded liposomes for pilosebaceous targeted delivery against alopecia areata. This study conducted the effect of charged liposomes on in vitro drug release, ex vivo skin permeation and drug retention behavior on rat skin. It was found that neutral liposomes showed maximum penetration and drug deposition in the pilosebaceous units compare to positively, negatively charged liposomes and non-liposomal formulation [170]. Monoclonal antibodies, DNA, vaccines in liposomes have also been studied for follicular pathway targeting [171–173].

Targeting the follicular pathway is still in its infancy and multiple safety studies should be carried out to prevent skin allergies or undesired systemic circulation of the drugs from the reservoirs in the sebaceous glands [174]. Still, the pilosebaceous unit is an important feature for topical delivery and should be studied further.

1.4.3 Pulmonary Targeted Drug Delivery

Respiratory diseases like asthma, tuberculosis, cystic fibrosis, lung cancer, and chronic obstructive pulmonary disease validate the use of delivering the drugs via lungs on top of which the anatomy and physiology of the respiratory system can be exploited for noninvasive, patient friendly systemic delivery of the drugs. The lungs provide a large surface area and a thin epithelial layer perfused with continuous blood flow. Targeting the lungs can provide quick systemic administration of the drugs and also assist in avoiding the first pass effect faced by oral drugs.

The pulmonary TDDS has to overcome barriers such as the mucus layer, alveolar lining fluid, epithelial cells, basement membrane, macrophages as well as enzymatic degradation. In the case of alveolar sacs the epithelial layer does not have tight junctions and as compared to the upper respiratory tract the rate of clearance is also less which makes them a good targeting location for drug/gene delivery [175, 176].

In treatment of diseases like asthma commonly used medications include anti-inflammatory drugs such as corticosteroids (beclomethasone, ciclesonide), beta-agonists (albuterol), anticholinergics delivered via inhalers and nebulizers. These long-term as well as immediate action systems are a preferred choice for several patients.

Current research in the pulmonary TDDS is highly concentrated on the use of nanocarriers especially liposomes and biodegradable polymeric nanoparticles. The important factors for efficient TDDS include size, shape and charge of the inhaled particles. For example, delivery to distal locations (lower respiratory tract) is favorable to particles with size around 1–5 μm, while larger particles will accumulate in the upper respiratory region. It has been noted that particles with size less than 1 μm are usually removed on exhalation [177]. Surface charge also plays a role

in how the particles interact after the inhalation as with low surface energy the particles will tend to aggregate less. The surface charge also determines the encapsulation efficiency of nanoparticles, their interaction with alveolar region and drug release [177]. Thus, optimization of physicochemical properties of the carriers forms a prime objective while developing the pulmonary TDDS.

Poly (DL-lactide-co-glycolide) (PLGA) microparticles containing rifampicin targeted against *Mycobacterium tuberculosis* infections were compared with "free" rifampicin in vivo in guinea pigs. The results not only highlighted that the aerosolized formulations reduced the infection but also that a single dose of the rifampicin in microparticles was comparable to daily doses of "free" rifampicin for 20 days [178]. In another study, treatment with aerosolized liposomal amphotericin B (AmBisome, Gilead Sciences, Foster City, California, USA) significantly improved survival compared to the aerosolized amphotericin B desoxycholate and placebo examined in immunocompromised rats with invasive pulmonary aspergillosis [179].

The group of Vyas [180, 181] carried out studies to develop aerosolized liposomes loaded with either rifampicin or amphotericin B against tuberculosis and aspergillosis infections respectively. The objective was to target the formulations to alveolar macrophages where the infection would be in densest form. The egg phosphatidylcholine (Egg PC) and cholesterol based liposomes were thus targeted to the alveolar macrophages by attaching macrophage-specific ligands (*O*-palmitoyl mannan, *O*-palmitoyl pullulan, *O*-steroyl amylopectin, and maleylated bovine serum albumin) or by imparting negative charges (with dicetylphosphate). Higher drug concentration in the lungs and preferential accumulation in the alveolar macrophages was observed in the targeted aerosolized formulations as compared to non-targeted liposomes as well as free drugs. Thus, Vyas highlighted the fact that ligand-attached liposomal aerosols had significant targeting potential.

Peptide and protein delivery is also studied as pulmonary TDDS with the benefit of large alveolar surface area and thin epithelium to aid absorption of the macromolecules. Perhaps, the most studied of such macromolecules is insulin. Multiple studies have been done with insulin-loaded microparticles to demonstrate efficient release in vitro and prolonged hypoglycemic effects in vivo in rats and guinea pigs [182–184]. It has also been shown that pulmonary TDDS can be utilized for gene delivery. A novel chitosan-based siRNA nanoparticle delivery system was developed by Howard et al. by complex formation between the siRNA and the chitosan polymers. The study of nasally delivered complexes demonstrated effective in vivo RNA interference in bronchiole epithelial cells of transgenic EGFP mice compared to the controls [185].

1.4.4 Retina

The drug delivery to the retina poses similar issues as brain drug delivery. This is because of the presence of the blood–retinal barrier (BRB), structurally similar to the BBB, which regulates the passage of the drugs to the retina from the blood.

The BRB too comprises tight junctions in its epithelium, a basement membrane as well as the presence of efflux transporter pumps such as P-glycoprotein (P-gp) [186, 187]. Thus, the delivery of drugs including small molecules, macromolecular peptides, and proteins is restricted into the retina. Targeted delivery to retina can comprise of physical targeting which are local (topical) and invasive methods and systemic targeting which is noninvasive where the therapeutic agents are administered systemically [188–190].

Topical administration involves the use of solutions and ointments as drug delivery systems and they are excellent choices for anterior segment of the eye and are patient friendly and cost appropriate too. But, these systems usually do not deliver drugs at effective levels at the retina which lies in the posterior parts of the eye [191, 192]. Along with that, the delivery systems are affected by drug loss due to washing off by tears, metabolism by the anterior segment enzymes and impermeability of the corneal epithelium [193]. Hence, as a route for targeted delivery to the retina, topical administration does not suit well.

Invasively administered drugs comprise intravitreal delivery, subconjunctival injections, and scleral implants. They avoid the barriers faced by the topical administration and are applied widely for targeted retinal delivery. Moreover, because the delivery is into the eye, systemic side effects are generally avoided. Definitely, the intravitreal injection is uncomfortable for the patient and frequent dosing is associated with high probabilities of injection associated infections and retinal detachment; still, advances are made to improve the dosing requirements and the drug presentation time at the retina by using nanocarriers and lipidic prodrugs [194–196]. Bourges et al. formulated polylactide (PLA) nanoparticles and observed targeted delivery and localization at the RPE cells after intravitreal injections in rats [197]. Another method is the use of intravitreal implants which can give sustained delivery for a longer period of time as compared to the injections; up to 6 months in case of implants compared to 2–3 times a week for injection. Such a delivery system is especially beneficial to patients with chronic eye disorders such as retinopathy. Vitrasert® is such an implant which is surgically inserted in the posterior region of the eye and delivers gancyclovir for up to 8 months. However, such inserts still carry the risk of loss of vision, vitreous haemorrhage, cataract formation and other adverse reactions [188]. Other invasive procedures employed include the scleral implants and subconjunctival injections which avoid the risk of retinal detachment associated with the procedures explained previously. Few studies have been carried out for scleral implants made from polymers such as poly (DL-lactide-co-glycolide) (PLGA) and ploy (DL-lactide) (PLA). Gancyclovir was delivered in these studies with sustained release of therapeutically effective doses obtained [198]. The subconjunctival injection beneath the conjunctiva enables the drugs to diffuse from there, through the sclera into the choroid [199].

In general the BRB is restrictive in allowing compounds to pass through except for nutrients. Hence, systemic administration results in very small amount of dose to reach the retina which is often below therapeutic levels. Higher dose administration results in systemic toxicity. Hyperosmotic mannitol injections can be employed to transiently disrupt the BRB and allow passage of the coadministered drug; but it

carries the risk of allowing infectious agents and toxins to pass through. Moreover the mannitol injections are responsible for BBB disruption too resulting in concurrent neurotoxicity. Now, the BRB has presence of transport systems similar to the BBB and they can be exploited for transport-mediated targeted drug delivery [190, 200]. Targeting ligands or antibodies can be attached to drug containing nanocarriers and prodrugs can also be made to achieve this purpose. Peptide transporters (PepTs) esp. PepT1 and PepT2 have been identified with broad substrate specificity towards dipeptides and tripeptides as well as peptidomimetics. PepT targeted 5′-amino acid ester prodrugs of nucleosides like gancyclovir, acyclovir, azidothymidine have driven increased bioavailability on oral administration [201]. Similarly, there has been observed presence of monocarboxylic acid transporters, folate transporters, and amino acid transporters on the BRB which can be utilized for targeted drug delivery [188, 190, 200].

1.4.5 Colon Targeted Drug Delivery

A number of diseases like inflammatory bowel diseases (IBD) like Crohn's disease and ulcerative colitis, colon cancer, irritable bowel syndrome (IBS), amoebiasis, etc. and desired transport of proteins and peptide drugs require the use of colon targeted drug delivery systems (CDDS). The general routes of reaching the colon are via the oral delivery or the rectal delivery. Using the rectal mode of administration is usually uncomfortable for the patient and can often result in irregular dose distribution. Conversely, using regular oral modes of delivery can degrade the drugs by acid actions in the stomach and alkaline and enzyme activity in the small intestine. Hence, for appropriate colon-specific delivery targeted systems should be utilized. So far, CDDS has seen the use of pH-dependent, time-dependent, and microflora-enzyme-dependent systems which have not proven to be foolproof. For example, it is possible that the pH-dependent system may survive the passage through the stomach but not the small intestine and the time-dependent system usually depends on the natural time for food and drug to passage through the gastrointestinal tract (GIT) which can be irregular esp. in the diseased states [202–204]. Improved technologies such as di-dependent systems utilize control by two factors to release the drug payload; for example, pH and time or pH and enzymes of microflora in the colon [205]. Ishibashi et al. developed three-layered capsule dosage form which consisted an acid-soluble polymer, a water-soluble polymer and an enteric polymer to deliver the active payload to the colon. Essentially this time- and pH-dependent dosage form was a predictable targeted system to deliver the drugs to the colon with high efficiency after in vitro and in vivo evaluations [206]. Yet another CDDS that depended on pH and microbes to deliver the drugs consisted of a traditional tablet core containing lactulose with additional layers of Eudragit E (acid soluble) and Eudragit L (enteric coat) on top of it, in that order, was developed to protect the active drug from the acid effects of the stomach (enteric coating), the alkaline pH of small intestine (acid soluble coating) and deliver to the colon wherein the lactulose would be

degraded by the colon bacteria. The enzymatic degradation of the lactulose would produce organic acids lowering the pH locally and dissolve the acid soluble coat releasing the drugs [207].

An alternative technique that can be used is to make prodrugs which provide protection in the upper GIT but undergoes enzymatic degradation in the colon to release the active drug. A study in the rats was performed using glycosidic prodrugs of dexamethasone and prednisolone. The prodrugs were not absorbed in the small intestine as they were hydrophilic thus reaching the colon intact. Once in the colon, the bacterial glycosidases cleaved the prodrugs to release the active drugs useful for targeting and treating the IBD in the colon. This study determined that the dexamethasone prodrug was better than the prednisolone prodrug [208, 209]. Moreover, it was suggested that modifications in the diet could induce the colon bacteria to produce specific enzymes which is a technique that can be used to further improve the efficiency of the delivery system. A large amount of interest has been seen in the development of azo-polymeric prodrugs to benefit from the azoreductase enzyme in the colon [210, 211]. Colazal® (Salix Pharmaceuticals Inc., North Carolina, USA) is an azo-prodrug of balsalazide indicated for ulcerative colitis.

The intrinsic ability of nanoparticles to accumulate at inflammation sites is also exploited for targeted delivery to the colon esp. in case of IBD. This results in long term deposition of the nanoparticles and drugs within at the site of inflammation [212].

1.4.6 Intracellular Targeting

In this section, the importance and strategies to carry out intracellular/subcellular targeting are highlighted. Once the therapeutics are able to reach the intended organ/tissue of action, they need to act either extracellularly or intracellularly. When the action is supposed to occur in extracellular regions, the task of arriving at the specific organ/tissue is enough. Yet when the mechanism of action of the therapeutic substance is on specific proteins, peptides, enzymes, nucleic acids (DNA/RNA) which are present within the cell, the TDDS needs to go a step or even two, in case of nuclear targeting, further to ensure that the specific drug/gene enter the cell and are in active form once they reach their intracellular targets. The targets in question may be located on the plasma membrane or cell components such as endosome, lysosome, endoplasmic reticulum, nucleus, mitochondria, or even mRNA binding complexes.

Plasma membrane targeting will be important for drugs whose actions are mediated through the proteins, lipids, signaling channels present on the plasma membrane. Targeting these drugs can facilitate high loading of the drugs around the cell increasing the effective concentrations where required. It also helps to reduce overall dose administered to the body. The plasma membrane has also been the subject of targeting in case of infections that depend on binding to the plasma membrane to initiate their life cycles. Fusion inhibitors class of HIV/AIDS drugs target and inhibit the HIV fusion and entry to the cells [213, 214].

The steps involved in intracellular targeting require the knowledge of how the cells can internalize components. Endocytosis is the process of absorbing molecules

by the cells, three ways of which are: phagocytosis, pinocytosis, and receptor-mediated endocytosis (RME). All of these processes lead into the endocytic pathway first step being the early endosomes responsible for sorting the internalized components and also mediating release of the receptors (in case of RME) for recycling. The early endosomes are characterized by mildly acidic pH. The early endosomes mature into the late endosomes or transfer the internalized components to the Golgi apparatus. Late endosomes also have mildly acidic pH (5.5) and result in formation of lysosomes after final sorting of the internalized material. Lysosomes are acidic and contain hydrolytic enzymes that degrade the material within. Definitely, the pathway consists of several signals and controls which are discussed in detail in this review [215]. Each of the steps in the endocytosis gives an opportunity to target.

Targeting the plasma membrane bound receptors (e.g., Tf receptor in tumor or brain) specifically allows binding to the intended cells of action which undergo RME and the TDDS are absorbed into the endosomes. They can be functionalized to endosomal markers to trigger the release of the drugs once inside the endosome. An acidic pH-sensitive system will, on entry into the endosomes, disintegrate to release the drug payload which can diffuse into the cytoplasm. Similarly, endosome-disrupting agents, which depend on the "proton sponge effect," can also be used to target the drugs/genes into the cells [216, 217]. The late endosome is responsible for trafficking the mannose-6-phosphate receptors and this can be a useful target for enzyme replacement therapy. Genetic disorders like Gaucher's and Fabry's require lysosomal enzyme replacement therapies where the mannose-6-phoshpate uptake can help target the enzyme replacements to the late endosomes and lysosomes [218]. Similarly, delivery of therapeutic substances to lysosomes was improved by lysosome-targeted nanosystems using lysosomotropic octadecyl-rhodamine B (RhB) [219, 220].

There are several drugs whose substrates lie in the cytoplasm. Even RNAi therapy requires that the siRNA be present in the cytoplasm to form the RNA-induced silencing complex (RISC). Hence, delivery of intact drug/gene to the cytoplasm is an important factor. As shown before, if the drug is internalized by endocytosis it is possible to initiate its release into the cytoplasm via endosome-disruption or stimulus responsive carriers. There is another technique which can deliver the drug/gene directly into the cytoplasm. It uses the cell penetrating peptides (CPPs) that transduce into the cells and directly release the payload into the cytosol. Several studies have been performed to explore this technology [221–224].

Multiple disease and disorders find their pathology to involve the role of mitochondria and its constituents. Consequently, drugs and nucleic acids with actions on mitochondria are useful bringing the question of targeting them to the mitochondria after entering the cytoplasm. A cytotoxic peptide (r7-kla) was made by conjugating mitochondrial membrane targeted fusion peptide (kla) with a cell-penetrating domain (r7) as an apoptosis inducer and an antitumor agent by causing targeted mitochondrial membrane disruption in both in vitro and in vivo experiments [225]. Other strategies may utilize similar targeting peptides for cell penetration and mitochondrial targeting to benefit patients of cancer as well as neurodegenerative diseases [226–232].

Gene therapy usually requires that the nucleic acids be delivered into the nucleus where the nuclear membrane forms an additional barrier. The usual approaches to gene therapy use viral-mediated as well as nonviral (e.g., liposomes) gene delivery which has been discussed in previous sections.

1.5 Summary

The field of medicine is filled with mires when it comes to safety and efficacy in applications of therapeutic substances to several diseases. Often the drug discovery provides potent leads but its research is not continued for the want of desirable physicochemical properties and absence of adverse effects. In case of some diseases such as cancer or HIV/AIDS it is not always possible to not use such drugs and while research of finding new class of drugs is ongoing, it is exciting to deliver the current drugs in a safe and efficacious fashion with targeted drug delivery systems. The advantage of such systems are to allow targeted deposition at intended site, sustained release, safety, reduced dosing frequency, and patient convenience. The goal of achieving efficient targeting has seen contributions from multiple fields like molecular biology, chemistry, and physics.

This chapter covered essential aspects of targeted drug delivery discussing challenges and strategies in several diseases and specific requirements of targeting at some locations in the body. Use of such strategies have led to improvement in disease conditions in several cases and underlines the importance of understanding the diseases, their physiology at tissue and molecular levels and identifying targets for developing TDDS.

While the TDDS has shown benefits in multiple conditions, further research is validated. The TDDS are not without pitfalls. For instance, while the targeting of cancer has shown several benefits in in vivo conditions, they are not able to completely cure cancer in humans. This is because cancer in humans is not just characterized by solid tumors, but metastatic cancer cells move around the body. Even if the solid tumors are targeted, the metastatic cancer cells may not be killed and cancer remission may be seen. Hence, targeting metastatic cells is also of prime importance. Other pitfalls attributed to targeting may include immune responses to antibody-directed therapies and the inability to achieve consistent pharmacokinetics when transferred from preclinical animal studies to clinical studies.

Still, the TDDS remain a viable approach to achieve efficacious treatments and continued exploration will lead to development of breakthrough therapies.

1.6 Conclusion

Thus, there exist both the variety of targets in the body and the variety of means to specifically bring pharmaceuticals to such targets. The past years have yielded significant preclinical data for several diseases. As we see throughout this chapter,

multiple studies have been carried out in vitro and in vivo to demonstrate the benefits of targeting drug delivery.

Major challenges exist in bringing TDDS from bench to bedside and continuous research needs to address them. One of the most important considerations is to understand the translation of preclinically proven TDDS into potential clinical material. Successful scale-up and industrial production of such systems, while keeping costs in check, will be the foremost step to see them in the clinics as well as individualized therapy.

The authors would like to highlight that this is an overview of different targeting strategies and would encourage readers to study each strategy in depth for better understanding.

References

1. Strebhardt K, Ullrich A (2008) Paul Ehrlich's magic bullet concept: 100 years of progress. Nat Rev Cancer 8(6):473–480
2. Langer R (2001) Drug delivery. Drugs on target. Science 293(5527):58–59
3. Childress EM, Kleinstreuer C, Kennedy AS (2012) A new catheter for tumor-targeting with radioactive microspheres in representative hepatic artery systems–part II: solid tumor-targeting in a patient-inspired hepatic artery system. J Biomech Eng 134(5):051005
4. Kleinstreuer C et al (2012) A new catheter for tumor targeting with radioactive microspheres in representative hepatic artery systems. Part I: impact of catheter presence on local blood flow and microsphere delivery. J Biomech Eng 134(5):051004
5. Dunaevsky A (2013) The gene-gun approach for transfection and labeling of cells in brain slices. Methods Mol Biol 1018:111–118
6. Han HK, Amidon GL (2000) Targeted prodrug design to optimize drug delivery. AAPS PharmSci 2(1):E6
7. Mahato R, Tai W, Cheng K (2011) Prodrugs for improving tumor targetability and efficiency. Adv Drug Deliv Rev 63(8):659–670
8. Torchilin VP (2006) Nanoparticulates as drug carriers. Imperial college press, London, UK
9. Garnett MC (2001) Targeted drug conjugates: principles and progress. Adv Drug Deliv Rev 53(2):171–216
10. Crielaard BJ et al (2012) Drug targeting systems for inflammatory disease: one for all, all for one. J Control Release 161(2):225–234
11. Stylianopoulos T (2013) EPR-effect: utilizing size-dependent nanoparticle delivery to solid tumors. Ther Deliv 4(4):421–423
12. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46(12 Pt 1):6387–6392
13. Torchilin V (2011) Tumor delivery of macromolecular drugs based on the EPR effect. Adv Drug Deliv Rev 63(3):131–135
14. Santos-Magalhaes NS, Mosqueira VC (2010) Nanotechnology applied to the treatment of malaria. Adv Drug Deliv Rev 62(4–5):560–575
15. Torchilin VP (2007) Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J 9(2):E128–E147
16. Zhang X, Lin Y, Gillies RJ (2010) Tumor pH and its measurement. J Nucl Med 51(8):1167–1170
17. Singh S, Khan AR, Gupta AK (2012) Role of glutathione in cancer pathophysiology and therapeutic interventions. J Exp Ther Oncol 9(4):303–316
18. Sawant RR et al (2012) Polyethyleneimine-lipid conjugate-based pH-sensitive micellar carrier for gene delivery. Biomaterials 33(15):3942–3951

19. Wu H, Zhu L, Torchilin VP (2013) pH-sensitive poly(histidine)-PEG/DSPE-PEG co-polymer micelles for cytosolic drug delivery. Biomaterials 34(4):1213–1222
20. Liu J et al (2011) Redox-responsive polyphosphate nanosized assemblies: a smart drug delivery platform for cancer therapy. Biomacromolecules 12(6):2407–2415
21. Pan YJ et al (2012) Redox/pH dual stimuli-responsive biodegradable nanohydrogels with varying responses to dithiothreitol and glutathione for controlled drug release. Biomaterials 33(27):6570–6579
22. Lee ES, Na K, Bae YH (2005) Doxorubicin loaded pH-sensitive polymeric micelles for reversal of resistant MCF-7 tumor. J Control Release 103(2):405–418
23. Veiseh O, Gunn JW, Zhang M (2010) Design and fabrication of magnetic nanoparticles for targeted drug delivery and imaging. Adv Drug Deliv Rev 62(3):284–304
24. Chomoucka J et al (2010) Magnetic nanoparticles and targeted drug delivering. Pharmacol Res 62(2):144–149
25. Kempe M et al (2010) The use of magnetite nanoparticles for implant-assisted magnetic drug targeting in thrombolytic therapy. Biomaterials 31(36):9499–9510
26. Bi F et al (2009) Chemical conjugation of urokinase to magnetic nanoparticles for targeted thrombolysis. Biomaterials 30(28):5125–5130
27. Chorny M et al (2010) Endothelial delivery of antioxidant enzymes loaded into non-polymeric magnetic nanoparticles. J Control Release 146(1):144–151
28. Riegler J et al (2010) Targeted magnetic delivery and tracking of cells using a magnetic resonance imaging system. Biomaterials 31(20):5366–5371
29. Pouponneau P et al (2011) Co-encapsulation of magnetic nanoparticles and doxorubicin into biodegradable microcarriers for deep tissue targeting by vascular MRI navigation. Biomaterials 32(13):3481–3486
30. Hua MY et al (2010) Magnetic-nanoparticle-modified paclitaxel for targeted therapy for prostate cancer. Biomaterials 31(28):7355–7363
31. Dilnawaz F et al (2010) Dual drug loaded superparamagnetic iron oxide nanoparticles for targeted cancer therapy. Biomaterials 31(13):3694–3706
32. Enriquez GG et al (2013) Formulation and evaluation of drug-loaded targeted magnetic microspheres for cancer therapy. Int J Nanomedicine 8:1393–1402
33. Hua MY et al (2011) Superhigh-magnetization nanocarrier as a doxorubicin delivery platform for magnetic targeting therapy. Biomaterials 32(34):8999–9010
34. Purushotham S, Ramanujan RV (2010) Thermoresponsive magnetic composite nanomaterials for multimodal cancer therapy. Acta Biomater 6(2):502–510
35. Wang H et al (2012) Folate-targeting magnetic core-shell nanocarriers for selective drug release and imaging. Int J Pharm 430(1–2):342–349
36. Dilnawaz F et al (2012) The transport of non-surfactant based paclitaxel loaded magnetic nanoparticles across the blood brain barrier in a rat model. Biomaterials 33(10):2936–2951
37. Yang FY et al (2012) Focused ultrasound and interleukin-4 receptor-targeted liposomal doxorubicin for enhanced targeted drug delivery and antitumor effect in glioblastoma multiforme. J Control Release 160(3):652–658
38. Park EJ et al (2012) Ultrasound-mediated blood-brain/blood-tumor barrier disruption improves outcomes with trastuzumab in a breast cancer brain metastasis model. J Control Release 163(3):277–284
39. Nomikou N, McHale AP (2010) Exploiting ultrasound-mediated effects in delivering targeted, site-specific cancer therapy. Cancer Lett 296(2):133–143
40. Nomikou N, Li YS, McHale AP (2010) Ultrasound-enhanced drug dispersion through solid tumours and its possible role in aiding ultrasound-targeted cancer chemotherapy. Cancer Lett 288(1):94–98
41. Gasselhuber A et al (2012) Targeted drug delivery by high intensity focused ultrasound mediated hyperthermia combined with temperature-sensitive liposomes: computational modelling and preliminary in vivovalidation. Int J Hyperthermia 28(4):337–348
42. Anderson CR et al (2011) Ultrasound molecular imaging of tumor angiogenesis with an integrin targeted microbubble contrast agent. Invest Radiol 46(4):215–224

43. Cochran MC et al (2011) Doxorubicin and paclitaxel loaded microbubbles for ultrasound triggered drug delivery. Int J Pharm 414(1–2):161–170
44. Wang CH et al (2012) Aptamer-conjugated and drug-loaded acoustic droplets for ultrasound theranosis. Biomaterials 33(6):1939–1947
45. Li P et al (2012) Ultrasound triggered drug release from 10-hydroxycamptothecin-loaded phospholipid microbubbles for targeted tumor therapy in mice. J Control Release 162(2):349–354
46. Zhao YZ et al (2011) Enhancing chemotherapeutic drug inhibition on tumor growth by ultrasound: an in vivo experiment. J Drug Target 19(2):154–160
47. Rapoport NY et al (2009) Controlled and targeted tumor chemotherapy by ultrasound-activated nanoemulsions/microbubbles. J Control Release 138(3):268–276
48. Ting CY et al (2012) Concurrent blood-brain barrier opening and local drug delivery using drug-carrying microbubbles and focused ultrasound for brain glioma treatment. Biomaterials 33(2):704–712
49. Korin N et al (2012) Shear-activated nanotherapeutics for drug targeting to obstructed blood vessels. Science 337(6095):738–742
50. Uesugi Y et al (2010) An ultrasound-responsive nano delivery system of tissue-type plasminogen activator for thrombolytic therapy. J Control Release 147(2):269–277
51. Sun L et al (2013) The use of cationic microbubbles to improve ultrasound-targeted gene delivery to the ischemic myocardium. Biomaterials 34(8):2107–2116
52. Deckers R, Moonen CT (2010) Ultrasound triggered, image guided, local drug delivery. J Control Release 148(1):25–33
53. Bohmer MR et al (2009) Ultrasound triggered image-guided drug delivery. Eur J Radiol 70(2):242–253
54. Rapoport N et al (2011) Ultrasound-mediated tumor imaging and nanotherapy using drug loaded, block copolymer stabilized perfluorocarbon nanoemulsions. J Control Release 153(1):4–15
55. Liu HL et al (2010) Magnetic resonance monitoring of focused ultrasound/magnetic nanoparticle targeting delivery of therapeutic agents to the brain. Proc Natl Acad Sci U S A 107(34):15205–15210
56. Ranjan A et al (2012) Image-guided drug delivery with magnetic resonance guided high intensity focused ultrasound and temperature sensitive liposomes in a rabbit Vx2 tumor model. J Control Release 158(3):487–494
57. Sharma SK et al (1994) Antibody-directed enzyme prodrug therapy (ADEPT). A three-phase study in ovarian tumor xenografts. Cell Biophys 24–25:219–228
58. Rajendran L, Knolker HJ, Simons K (2010) Subcellular targeting strategies for drug design and delivery. Nat Rev Drug Discov 9(1):29–42
59. Christopher ME, Wong JP (2006) Recent developments in delivery of nucleic acid-based antiviral agents. Curr Pharm Des 12(16):1995–2006
60. Shan L et al (2012) A paclitaxel-conjugated adenovirus vector for targeted drug delivery for tumor therapy. Biomaterials 33(1):146–162
61. Musacchio T et al (2010) Effective stabilization and delivery of siRNA: reversible siRNA-phospholipid conjugate in nanosized mixed polymeric micelles. Bioconjug Chem 21(8): 1530–1536
62. Buyens K et al (2012) Liposome based systems for systemic siRNA delivery: stability in blood sets the requirements for optimal carrier design. J Control Release 158(3):362–370
63. Goula D et al (1998) Polyethylenimine-based intravenous delivery of transgenes to mouse lung. Gene Ther 5(9):1291–1295
64. Patel NR et al (2013) Nanopreparations to overcome multidrug resistance in cancer. Adv Drug Deliv Rev 65(13–14):1748–1762
65. Grupp SA et al (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368(16):1509–1518
66. Maeda H, Bharate GY, Daruwalla J (2009) Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur J Pharm Biopharm 71(3):409–419

67. Patil YB et al (2010) The use of nanoparticle-mediated targeted gene silencing and drug delivery to overcome tumor drug resistance. Biomaterials 31(2):358–365
68. Patel NR et al (2011) Reversal of multidrug resistance by co-delivery of tariquidar (XR9576) and paclitaxel using long-circulating liposomes. Int J Pharm 416(1):296–299
69. Bae S et al (2012) Doxorubicin-loaded human serum albumin nanoparticles surface-modified with TNF-related apoptosis-inducing ligand and transferrin for targeting multiple tumor types. Biomaterials 33(5):1536–1546
70. Wagner S et al (2010) Enhanced drug targeting by attachment of an anti alphav integrin antibody to doxorubicin loaded human serum albumin nanoparticles. Biomaterials 31(8): 2388–2398
71. Sawant RR et al (2013) Targeted transferrin-modified polymeric micelles: enhanced efficacy in vitro and in vivo in ovarian carcinoma. Mol Pharm 11(2):375–381
72. Lukyanov AN et al (2004) Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anti-cancer antibody. J Control Release 100(1):135–144
73. Abouzeid AH et al (2013) Anti-cancer activity of anti-GLUT1 antibody-targeted polymeric micelles co-loaded with curcumin and doxorubicin. J Drug Target 21(10):994–1000
74. Zhu L, Torchilin VP (2013) Stimulus-responsive nanopreparations for tumor targeting. Integr Biol (Camb) 5(1):96–107
75. Koren E et al (2012) Multifunctional PEGylated 2C5-immunoliposomes containing pH-sensitive bonds and TAT peptide for enhanced tumor cell internalization and cytotoxicity. J Control Release 160(2):264–273
76. Sawant RR, Torchilin VP (2011) Design and synthesis of novel functional lipid-based bioconjugates for drug delivery and other applications. Methods Mol Biol 751:357–378
77. Perche F, Torchilin VP (2013) Recent trends in multifunctional liposomal nanocarriers for enhanced tumor targeting. J Drug Deliv 2013:705265
78. Torchilin VP (2006) Multifunctional nanocarriers. Adv Drug Deliv Rev 58(14):1532–1555
79. Jackman AL, Leamon CP (2011) Targeted drug strategies for cancer and inflammation. Springer, New York, NY
80. Kratz F, Senter P, Steinhagen H (2012) Drug delivery in oncology: from basic research to cancer therapy. Weinheim, Wiley-VCH-Verlag
81. Neidle S (2013) Cancer drug design and discovery. Elsevier Science, Amsterdam
82. Tew K, Fisher P (2013) Advances in cancer research. Elsevier Science, Amsterdam
83. Giaccone G, Soria JC (2013) Targeted therapies in oncology. Taylor & Francis, London
84. Antoniadou A et al (2013) Impact of a hospital-wide antibiotic restriction policy program on the resistance rates of nosocomial Gram-negative bacteria. Scand J Infect Dis 45(6):438–445
85. Hurdle JG et al (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9(1):62–75
86. Sharma A et al (2012) Nano-technology for targeted drug delivery to combat antibiotic resistance. Expert Opin Drug Deliv 9(11):1325–1332
87. Zhang L et al (2010) Development of nanoparticles for antimicrobial drug delivery. Curr Med Chem 17(6):585–594
88. Takemoto K et al (2004) Comparative studies on the efficacy of Am Bisome and Fungizone in a mouse model of disseminated aspergillosis. J Antimicrob Chemother 53(2):311–317
89. Umamaheshwari RB, Jain NK (2003) Receptor mediated targeting of lectin conjugated gliadin nanoparticles in the treatment of Helicobacter pylori. J Drug Target 11(7):415–423, discussion 423–424
90. Subramanya S et al (2010) Targeted delivery of small interfering RNA to human dendritic cells to suppress dengue virus infection and associated proinflammatory cytokine production. J Virol 84(5):2490–2501
91. Koul A et al (2011) The challenge of new drug discovery for tuberculosis. Nature 469(7331):483–490
92. Kaneko T, Cooper C, Mdluli K (2011) Challenges and opportunities in developing novel drugs for TB. Future Med Chem 3(11):1373–1400

93. Clemens DL et al (2012) Targeted intracellular delivery of antituberculosis drugs to Mycobacterium tuberculosis-infected macrophages via functionalized mesoporous silica nanoparticles. Antimicrob Agents Chemother 56(5):2535–2545
94. Bhatt K, Salgame P (2007) Host innate immune response to Mycobacterium tuberculosis. J Clin Immunol 27(4):347–362
95. Chono S et al (2008) Efficient drug targeting to rat alveolar macrophages by pulmonary administration of ciprofloxacin incorporated into mannosylated liposomes for treatment of respiratory intracellular parasitic infections. J Control Release 127(1):50–58
96. Wijagkanalan W et al (2008) Efficient targeting to alveolar macrophages by intratracheal administration of mannosylated liposomes in rats. J Control Release 125(2):121–130
97. Pandey R, Khuller GK (2005) Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis. Tuberculosis (Edinb) 85(4):227–234
98. Abdulla S et al (2013) Randomized, controlled trial of the long term safety, immunogenicity and efficacy of RTS, S/AS02(D) malaria vaccine in infants living in a malaria-endemic region. Malar J 12:11
99. Qiu L, Jing N, Jin Y (2008) Preparation and in vitro evaluation of liposomal chloroquine diphosphate loaded by a transmembrane pH-gradient method. Int J Pharm 361(1–2): 56–63
100. Robertson RT et al (2008) Liposomes incorporating a Plasmodium amino acid sequence target heparan sulfate binding sites in liver. J Pharm Sci 97(8):3257–3273
101. Haynes SM et al (2008) Liposomal polyethyleneglycol and polyethyleneglycol-peptide combinations for active targeting to liver in vivo. Drug Deliv 15(4):207–217
102. Tsai MS et al (2011) Binding patterns of peptide-containing liposomes in liver and spleen of developing mice: comparison with heparan sulfate immunoreactivity. J Drug Target 19(7):506–515
103. Mosqueira VC et al (2004) Efficacy and pharmacokinetics of intravenous nanocapsule formulations of halofantrine in Plasmodium berghei-infected mice. Antimicrob Agents Chemother 48(4):1222–1228
104. Gupta Y, Jain A, Jain SK (2007) Transferrin-conjugated solid lipid nanoparticles for enhanced delivery of quinine dihydrochloride to the brain. J Pharm Pharmacol 59(7):935–940
105. Agrawal AK, Gupta CM (2000) Tuftsin-bearing liposomes in treatment of macrophage-based infections. Adv Drug Deliv Rev 41(2):135–146
106. Olotu A et al (2013) Four-year efficacy of RTS, S/AS01E and its interaction with malaria exposure. N Engl J Med 368(12):1111–1120
107. Moorthy VS et al (2013) Assessment of the RTS, S/AS01 malaria vaccine. Lancet Infect Dis 13(4):280–282
108. Schrager LK, D'Souza MP (1998) Cellular and anatomical reservoirs of HIV-1 in patients receiving potent antiretroviral combination therapy. JAMA 280(1):67–71
109. Gupta U, Jain NK (2010) Non-polymeric nano-carriers in HIV/AIDS drug delivery and targeting. Adv Drug Deliv Rev 62(4–5):478–490
110. Taylor PR et al (2005) Macrophage receptors and immune recognition. Annu Rev Immunol 23:901–944
111. Mishra V et al (2006) Targeted brain delivery of AZT via transferrin anchored pegylated albumin nanoparticles. J Drug Target 14(1):45–53
112. Kaur CD, Nahar M, Jain NK (2008) Lymphatic targeting of zidovudine using surface-engineered liposomes. J Drug Target 16(10):798–805
113. Shah LK, Amiji MM (2006) Intracellular delivery of saquinavir in biodegradable polymeric nanoparticles for HIV/AIDS. Pharm Res 23(11):2638–2645
114. Gunaseelan S et al (2010) Surface modifications of nanocarriers for effective intracellular delivery of anti-HIV drugs. Adv Drug Deliv Rev 62(4–5):518–531
115. das Neves J et al (2010) Nanotechnology-based systems for the treatment and prevention of HIV/AIDS. Adv Drug Deliv Rev 62(4–5):458–477
116. Wong HL et al (2010) Nanotechnology applications for improved delivery of antiretroviral drugs to the brain. Adv Drug Deliv Rev 62(4–5):503–517

117. Alam MI et al (2010) Strategy for effective brain drug delivery. Eur J Pharm Sci 40(5):385–403
118. Pardridge WM (2003) Blood-brain barrier drug targeting: the future of brain drug development. Mol Interv 3(2):90, -105, 51
119. Roney C et al (2005) Targeted nanoparticles for drug delivery through the blood-brain barrier for Alzheimer's disease. J Control Release 108(2–3):193–214
120. Westphal M et al (2006) Gliadel wafer in initial surgery for malignant glioma: long-term follow-up of a multicenter controlled trial. Acta Neurochir (Wien) 148(3):269–275, discussion 275
121. Beduneau A, Saulnier P, Benoit JP (2007) Active targeting of brain tumors using nanocarriers. Biomaterials 28(33):4947–4967
122. Temsamani J et al (2000) Brain drug delivery technologies: novel approaches for transporting therapeutics. Pharm Sci Technolo Today 3(5):155–162
123. Begley DJ (2004) Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther 104(1):29–45
124. Wang M, Etu J, Joshi S (2007) Enhanced disruption of the blood brain barrier by intracarotid mannitol injection during transient cerebral hypoperfusion in rabbits. J Neurosurg Anesthesiol 19(4):249–256
125. Cosolo WC et al (1989) Blood-brain barrier disruption using mannitol: time course and electron microscopy studies. Am J Physiol 256(2 Pt 2):R443–R447
126. Doolittle ND et al (1998) Blood-brain barrier disruption for the treatment of malignant brain tumors: The National Program. J Neurosci Nurs 30(2):81–90
127. Erdlenbruch B et al (2002) Increased delivery of erucylphosphocholine to C6 gliomas by chemical opening of the blood-brain barrier using intracarotid pentylglycerol in rats. Cancer Chemother Pharmacol 50(4):299–304
128. Cloughesy TF, Black KL (1995) Pharmacological blood-brain barrier modification for selective drug delivery. J Neurooncol 26(2):125–132
129. Salahuddin TS et al (1988) Observations on exsudation of fibronectin, fibrinogen and albumin in the brain after carotid infusion of hyperosmolar solutions. An immunohistochemical study in the rat indicating longlasting changes in the brain microenvironment and multifocal nerve cell injuries. Acta Neuropathol 76(1):1–10
130. Sawynok J (1986) The therapeutic use of heroin: a review of the pharmacological literature. Can J Physiol Pharmacol 64(1):1–6
131. Yang SC et al (1999) Body distribution in mice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on brain. J Control Release 59(3):299–307
132. Tsuji A (2005) Small molecular drug transfer across the blood-brain barrier via carrier-mediated transport systems. NeuroRx 2(1):54–62
133. Mena I, Cotzias GC (1975) Protein intake and treatment of Parkinson's disease with levodopa. N Engl J Med 292(4):181–184
134. Pardridge WM (1992) Recent developments in peptide drug delivery to the brain. Pharmacol Toxicol 71(1):3–10
135. Pardridge WM (2001) Brain drug targeting and gene technologies. Jpn J Pharmacol 87(2): 97–103
136. Thole M et al (2002) Uptake of cationzied albumin coupled liposomes by cultured porcine brain microvessel endothelial cells and intact brain capillaries. J Drug Target 10(4):337–344
137. Vorbrodt AW (1989) Ultracytochemical characterization of anionic sites in the wall of brain capillaries. J Neurocytol 18(3):359–368
138. Bickel U et al (1994) In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. J Histochem Cytochem 42(11):1493–1497
139. Shi N, Pardridge WM (2000) Noninvasive gene targeting to the brain. Proc Natl Acad Sci U S A 97(13):7567–7572
140. Gosk S et al (2004) Targeting anti-transferrin receptor antibody (OX26) and OX26-conjugated liposomes to brain capillary endothelial cells using in situ perfusion. J Cereb Blood Flow Metab 24(11):1193–1204

141. Beduneau A et al (2007) Design of targeted lipid nanocapsules by conjugation of whole antibodies and antibody Fab′ fragments. Biomaterials 28(33):4978–4990
142. Pardridge WM (2010) Biopharmaceutical drug targeting to the brain. J Drug Target 18(3):157–167
143. Pardridge WM et al (1995) Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries in vitro and rapid transcytosis through the blood-brain barrier in vivo in the primate. Pharm Res 12(6):807–816
144. Dehouck B et al (1994) Upregulation of the low density lipoprotein receptor at the blood-brain barrier: intercommunications between brain capillary endothelial cells and astrocytes. J Cell Biol 126(2):465–473
145. Meresse S et al (1989) Low-density lipoprotein receptor on endothelium of brain capillaries. J Neurochem 53(2):340–345
146. Ramge P et al (2000) Polysorbate-80 coating enhances uptake of polybutylcyanoacrylate (PBCA)-nanoparticles by human and bovine primary brain capillary endothelial cells. Eur J Neurosci 12(6):1931–1940
147. Alyautdin RN et al (1997) Delivery of loperamide across the blood-brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. Pharm Res 14(3):325–328
148. Ambruosi A et al (2006) Biodistribution of polysorbate 80-coated doxorubicin-loaded [14C]-poly(butyl cyanoacrylate) nanoparticles after intravenous administration to glioblastoma-bearing rats. J Drug Target 14(2):97–105
149. Wilson B et al (2008) Targeted delivery of tacrine into the brain with polysorbate 80-coated poly(n-butylcyanoacrylate) nanoparticles. Eur J Pharm Biopharm 70(1):75–84
150. Wilson B et al (2008) Poly(n-butylcyanoacrylate) nanoparticles coated with polysorbate 80 for the targeted delivery of rivastigmine into the brain to treat Alzheimer's disease. Brain Res 1200:159–168
151. Blasi P et al (2007) Solid lipid nanoparticles for targeted brain drug delivery. Adv Drug Deliv Rev 59(6):454–477
152. Salem AK et al (2001) Synthesis and characterisation of a degradable poly(lactic acid)-poly(ethylene glycol) copolymer with biotinylated end groups. Biomacromolecules 2(2):575–580
153. Prow TW et al (2011) Nanoparticles and microparticles for skin drug delivery. Adv Drug Deliv Rev 63(6):470–491
154. Papakostas D et al (2011) Nanoparticles in dermatology. Arch Dermatol Res 303(8): 533–550
155. Agrawal U et al (2013) Hyperbranched dendritic nano-carriers for topical delivery of dithranol. J Drug Target 21(5):497–506
156. Bhalekar MR et al (2009) Preparation and evaluation of miconazole nitrate-loaded solid lipid nanoparticles for topical delivery. AAPS PharmSciTech 10(1):289–296
157. Liu J et al (2007) Isotretinoin-loaded solid lipid nanoparticles with skin targeting for topical delivery. Int J Pharm 328(2):191–195
158. Maia CS, Mehnert W, Schafer-Korting M (2000) Solid lipid nanoparticles as drug carriers for topical glucocorticoids. Int J Pharm 196(2):165–167
159. Tang T et al (2011) Targeting superficial or nodular Basal cell carcinoma with topically formulated small molecule inhibitor of smoothened. Clin Cancer Res 17(10):3378–3387
160. de Jalon EG et al (2001) PLGA microparticles: possible vehicles for topical drug delivery. Int J Pharm 226(1–2):181–184
161. Pierre MB, Dos Santos Miranda Costa I (2011) Liposomal systems as drug delivery vehicles for dermal and transdermal applications. Arch Dermatol Res 303(9):607–621
162. Knorr F et al (2009) Follicular transport route–research progress and future perspectives. Eur J Pharm Biopharm 71(2):173–180
163. Lademann J et al (2001) Investigation of follicular penetration of topically applied substances. Skin Pharmacol Appl Skin Physiol 14(Suppl 1):17–22
164. Lademann J et al (2008) Hair follicles–an efficient storage and penetration pathway for topically applied substances. Summary of recent results obtained at the Center of Experimental

and Applied Cutaneous Physiology, Charite -Universitatsmedizin Berlin, Germany. Skin Pharmacol Physiol 21(3):150–155
165. Toll R et al (2004) Penetration profile of microspheres in follicular targeting of terminal hair follicles. J Invest Dermatol 123(1):168–176
166. Meidan VM, Bonner MC, Michniak BB (2005) Transfollicular drug delivery–is it a reality? Int J Pharm 306(1–2):1–14
167. Blume-Peytavi U, Vogt A (2011) Human hair follicle: reservoir function and selective targeting. Br J Dermatol 165(Suppl 2):13–17
168. Otberg N et al (2007) Follicular penetration of topically applied caffeine via a shampoo formulation. Skin Pharmacol Physiol 20(4):195–198
169. Agarwal R, Katare OP, Vyas SP (2000) The pilosebaceous unit: a pivotal route for topical drug delivery. Methods Find Exp Clin Pharmacol 22(2):129–133
170. Jain B et al (2010) Development and characterization of minoxidil-loaded liposomal system for delivery to pilosebaceous units. J Liposome Res 20(2):105–114
171. Balsari AL et al (1994) Protection against doxorubicin-induced alopecia in rats by liposome-entrapped monoclonal antibodies. FASEB J 8(2):226–230
172. Yarosh D et al (1994) Localization of liposomes containing a DNA repair enzyme in murine skin. J Invest Dermatol 103(4):461–468
173. Gupta PN et al (2005) Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. Int J Pharm 293(1–2):73–82
174. Wosicka H, Cal K (2010) Targeting to the hair follicles: current status and potential. J Dermatol Sci 57(2):83–89
175. Rytting E et al (2008) Biodegradable polymeric nanocarriers for pulmonary drug delivery. Expert Opin Drug Deliv 5(6):629–639
176. Vyas SP, Khatri K (2007) Liposome-based drug delivery to alveolar macrophages. Expert Opin Drug Deliv 4(2):95–99
177. Shoyele SA, Cawthorne S (2006) Particle engineering techniques for inhaled biopharmaceuticals. Adv Drug Deliv Rev 58(9–10):1009–1029
178. Garcia-Contreras L et al (2006) Evaluation of dosing regimen of respirable rifampicin biodegradable microspheres in the treatment of tuberculosis in the guinea pig. J Antimicrob Chemother 58(5):980–986
179. Ruijgrok EJ, Vulto AG, Van Etten EW (2001) Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats. J Antimicrob Chemother 48(1):89–95
180. Vyas SP et al (2005) Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages. Int J Pharm 296(1–2):12–25
181. Vyas SP et al (2004) Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. Int J Pharm 269(1):37–49
182. Zhang Q, Shen Z, Nagai T (2001) Prolonged hypoglycemic effect of insulin-loaded polybutylcyanoacrylate nanoparticles after pulmonary administration to normal rats. Int J Pharm 218(1–2):75–80
183. Kawashima Y et al (1999) Pulmonary delivery of insulin with nebulized DL-lactide/glycolide copolymer (PLGA) nanospheres to prolong hypoglycemic effect. J Control Release 62(1–2):279–287
184. Amidi M et al (2008) Preparation and physicochemical characterization of supercritically dried insulin-loaded microparticles for pulmonary delivery. Eur J Pharm Biopharm 68(2):191–200
185. Howard KA et al (2006) RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. Mol Ther 14(4):476–484
186. Kennedy BG, Mangini NJ (2002) P-glycoprotein expression in human retinal pigment epithelium. Mol Vis 8:422–430
187. Marmor MF (1975) Structure and function of the retinal pigment epithelium. Int Ophthalmol Clin 15(1):115–130
188. Duvvuri S, Majumdar S, Mitra AK (2003) Drug delivery to the retina: challenges and opportunities. Expert Opin Biol Ther 3(1):45–56

189. Urtti A (2006) Challenges and obstacles of ocular pharmacokinetics and drug delivery. Adv Drug Deliv Rev 58(11):1131–1135
190. Janoria KG et al (2007) Novel approaches to retinal drug delivery. Expert Opin Drug Deliv 4(4):371–388
191. Lee SS, Hughes PM, Robinson MR (2009) Recent advances in drug delivery systems for treating ocular complications of systemic diseases. Curr Opin Ophthalmol 20(6):511–519
192. Geroski DH, Edelhauser HF (2000) Drug delivery for posterior segment eye disease. Invest Ophthalmol Vis Sci 41(5):961–964
193. Kaur IP, Kanwar M (2002) Ocular preparations: the formulation approach. Drug Dev Ind Pharm 28(5):473–493
194. Diaz-Llopis M et al (1992) Liposomally-entrapped ganciclovir for the treatment of cytomegalovirus retinitis in AIDS patients. Eperimental toxicity and pharmacokinetics, and clinical trial. Doc Ophthalmol 82(4):297–305
195. Veloso AA Jr et al (1997) Ganciclovir-loaded polymer microspheres in rabbit eyes inoculated with human cytomegalovirus. Invest Ophthalmol Vis Sci 38(3):665–675
196. Maurice D (2001) Review: practical issues in intravitreal drug delivery. J Ocul Pharmacol Ther 17(4):393–401
197. Bourges JL et al (2003) Ocular drug delivery targeting the retina and retinal pigment epithelium using polylactide nanoparticles. Invest Ophthalmol Vis Sci 44(8):3562–3569
198. Sakurai E et al (2001) Scleral plug of biodegradable polymers containing ganciclovir for experimental cytomegalovirus retinitis. Invest Ophthalmol Vis Sci 42(9):2043–2048
199. Kim TW et al (2002) Intraocular distribution of 70-kDa dextran after subconjunctival injection in mice. Invest Ophthalmol Vis Sci 43(6):1809–1816
200. Hosoya K, Tachikawa M (2009) Inner blood-retinal barrier transporters: role of retinal drug delivery. Pharm Res 26(9):2055–2065
201. Han H et al (1998) 5′-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. Pharm Res 15(8):1154–1159
202. McConnell EL, Short MD, Basit AW (2008) An in vivo comparison of intestinal pH and bacteria as physiological trigger mechanisms for colonic targeting in man. J Control Release 130(2):154–160
203. Matsuda K et al (1996) Effect of food intake on the delivery of fluorescein as a model drug in colon delivery capsule after oral administration to beagle dogs. J Drug Target 4(2):59–67
204. Patel MM (2011) Cutting-edge technologies in colon-targeted drug delivery systems. Expert Opin Drug Deliv 8(10):1247–1258
205. Philip AK, Philip B (2010) Colon targeted drug delivery systems: a review on primary and novel approaches. Oman Med J 25(2):79–87
206. Ishibashi T et al (1999) In vivo drug release behavior in dogs from a new colon-targeted delivery system. J Control Release 57(1):45–53
207. Yang L, Chu JS, Fix JA (2002) Colon-specific drug delivery: new approaches and in vitro/in vivo evaluation. Int J Pharm 235(1–2):1–15
208. Friend DR, Chang GW (1985) Drug glycosides: potential prodrugs for colon-specific drug delivery. J Med Chem 28(1):51–57
209. Friend DR, Chang GW (1984) A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria. J Med Chem 27(3):261–266
210. Saffran M et al (1991) Oral insulin in diabetic dogs. J Endocrinol 131(2):267–278
211. Shantha KL, Ravichandran P, Rao KP (1995) Azo polymeric hydrogels for colon targeted drug delivery. Biomaterials 16(17):1313–1318
212. Lamprecht A et al (2001) Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease. J Pharmacol Exp Ther 299(2):775–781
213. Eckert DM, Kim PS (2001) Mechanisms of viral membrane fusion and its inhibition. Annu Rev Biochem 70:777–810
214. Zhou G, Chu S (2013) Discovery of small molecule fusion inhibitors targeting HIV-1 gp41. Curr Pharm Des 19(10):1818–1826
215. Gruenberg J (2001) The endocytic pathway: a mosaic of domains. Nat Rev Mol Cell Biol 2(10):721–730

216. Duan H, Nie S (2007) Cell-penetrating quantum dots based on multivalent and endosome-disrupting surface coatings. J Am Chem Soc 129(11):3333–3338
217. Suma T et al (2012) Smart multilayered assembly for biocompatible siRNA delivery featuring dissolvable silica, endosome-disrupting polycation, and detachable PEG. ACS Nano 6(8):6693–6705
218. Grabowski GA, Hopkin RJ (2003) Enzyme therapy for lysosomal storage disease: principles, practice, and prospects. Annu Rev Genomics Hum Genet 4:403–436
219. Koshkaryev A et al (2011) Targeting of lysosomes by liposomes modified with octadecyl-rhodamine B. J Drug Target 19(8):606–614
220. Thekkedath R, Koshkaryev A, Torchilin VP (2013) Lysosome-targeted octadecyl-rhodamine B-liposomes enhance lysosomal accumulation of glucocerebrosidase in Gaucher's cells in vitro. Nanomedicine (Lond) 8(7):1055–1065
221. Torchilin VP (2007) Tatp-mediated intracellular delivery of pharmaceutical nanocarriers. Biochem Soc Trans 35(Pt 4):816–820
222. Sawant RR, Torchilin VP (2009) Enhanced cytotoxicity of TATp-bearing paclitaxel-loaded micelles in vitro and in vivo. Int J Pharm 374(1–2):114–118
223. Kale AA, Torchilin VP (2007) "Smart" drug carriers: PEGylated TATp-modified pH-sensitive liposomes. J Liposome Res 17(3–4):197–203
224. Patel LN, Zaro JL, Shen WC (2007) Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. Pharm Res 24(11):1977–1992
225. Law B et al (2006) A mitochondrial targeted fusion peptide exhibits remarkable cytotoxicity. Mol Cancer Ther 5(8):1944–1949
226. Muratovska A et al (2001) Targeting large molecules to mitochondria. Adv Drug Deliv Rev 49(1–2):189–198
227. Szeto HH (2006) Mitochondria-targeted peptide antioxidants: novel neuroprotective agents. AAPS J 8(3):E521–E531
228. Szeto HH (2008) Development of mitochondria-targeted aromatic-cationic peptides for neurodegenerative diseases. Ann N Y Acad Sci 1147:112–121
229. Biswas S et al (2012) Liposomes loaded with paclitaxel and modified with novel triphenylphosphonium-PEG-PE conjugate possess low toxicity, target mitochondria and demonstrate enhanced antitumor effects in vitro and in vivo. J Control Release 159(3):393–402
230. Patel NR et al (2010) Mitochondria-targeted liposomes improve the apoptotic and cytotoxic action of sclareol. J Liposome Res 20(3):244–249
231. Biswas S et al (2012) Surface conjugation of triphenylphosphonium to target poly(amidoamine) dendrimers to mitochondria. Biomaterials 33(18):4773–4782
232. Biswas S et al (2011) Surface modification of liposomes with rhodamine-123-conjugated polymer results in enhanced mitochondrial targeting. J Drug Target 19(7):552–561

Part II
Disease-Based Targeting

Chapter 2
Recent Advances in Tumor Targeting Approaches

Kaushik Thanki, Varun Kushwah, and Sanyog Jain

Abbreviations

17 AAG	17-Allylamino-17-Demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamine-17-demethoxy-geldanamycin
ADCC	Antibody dependent cellular cytotoxicity
ADEPT	Antibody directed enzyme prodrug therapy
ANA	Monoclonal antinuclear autoantibody
ASM	Acid sphingomyelinase
ATP	Adenosine triphosphate
BCS	Biopharmaceutical classification system
BET	Bromodomain and extra-terminal
BP	Binding protein
BRCA	Breast cancer gene
CDC	Complement-activation dependent cytotoxicity
CDKs	Cyclin dependent kinases
CNS	Central nervous system
CTLA	Cytotoxic T-lymphocyte antigen
dgRTA	Deglycosylated ricin A chain
DMXAA	5,6-Dimethylxanthenone-4-acetic acid
DNA	Deoxy ribose nucleic acid
DOTA	d-Tyr-d-Lys(HSG)-d-Glu-d-Lys(HSG)-NH_2
EBRT	External-beam radiation therapy
ECM	Extracellular matrix
EPCs	Endothelial precursor cells

K. Thanki • V. Kushwah • S. Jain (✉)
Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Mohali, Punjab, India
e-mail: sanyogjain@niper.ac.in; sanyogjain@rediffmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_2

EZH2	Enhancer of Zeste homolog 2
FAK	Focal adhesion kinase
FDG	2-Deoxy-2-(^{18}F) fluoro-D-glucose
GMCSF	Granulocyte-macrophage colony-stimulating factor
GRP78	78 kDa glucose-regulated protein
H3K4me3/2	Trimethylation and dimethylation of histone H3 at lysine 4
HAMA	Human anti-mouse antibody
HARA	Human anti-ricin antibody
hCG	Human chorionic gonadotrophin
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia inducible factor
HOP	HSP90 organizing protein
HPMA	N-(2-hydroxy propyl) methacrylamide
HSP	Heat shock proteins
IFP	Interstitial fluid pressure
IL	Interleukin
IMP-288	1,4,7,10-Tetraazacyclododecane-N,N′,N″,N′″-tetraacetic acid
JAG1	Jagged 1 protein
LECs	Lymphatic endothelial cells
LLC	Lewis lung carcinoma
LTTs	Ligand-targeted therapeutics
mAbs	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporters
MMPs	Matrix metalloproteinases
MPS	Macrophagocytosis systems
mTOR	Mechanistic target of rapamycin
Myc	Myelocytomatosis oncogene
p14^{ARF}	Alternate reading frame
PI-3	Phosphoinositide 3-kinase
PDEPT	Polymer directed enzyme prodrug therapy
PE	Phosphatidyl ethanolamine
PKB	Protein kinase B
PSMC2	26S protease regulatory subunit 7 gene
pRb	Retinoblastoma tumor suppressor protein
RAIT	Radioimmunotherapy
Rb	Retinoblastoma
RBC	Red blood cell
RES	Reticuloendothelial system
SAR	Structure–activity relationship
STA	3-(2,4-dihydroxy-5-isopropyl-phenyl)-4-(1-methyl-indol-5-yl)-5-hydroxy-[1,2,4]triazole
TCMC	2-(4-isothiocyanotobenzyl)-1, 4, 7, 10-tetraaza-1, 4, 7, 10-tetra-(2-carbamonyl methyl)-cyclododecane

TNF	Tumor necrosis factors
TP53/p53	Tumor protein P53
VEGF	Vascular endothelial growth factor
vSMCs	Vascular smooth muscle cells
WHO	World Health Organization

2.1 Introduction

Tremendous technological developments in the field of cancer therapy have been observed in the past few decades to combat the ever-increasing mortality rate and its peculiar pathophysiology, referred to as carcinogenesis. As per WHO records, cancer is a leading cause of death across the globe accounting 8.2 million and 14 million new cases in 2012 with almost twofold rise in next couple of decades [1]. By definition, cancer is referred to as a generic terminology covering over 200 different types of cancers. Crudely, it is a pathophysiological condition in which the normal cells transform into immortal cells that grow without any control, often referred to as carcinogenesis. Principally, persistent tissue injury and/or genetic factors such as mutations, epigenetic and global transcriptome changes contribute to carcinogenesis (Fig. 2.1). Cumulatively, it could be considered as multistep (comprising a variety of genetic alterations), multipath (including various apoptotic and angiogenesis pathways), and multifocal (constitutive of both field carcinogenesis and clonal expansion) [2]. Subsequently these changes lead to distinct tumor

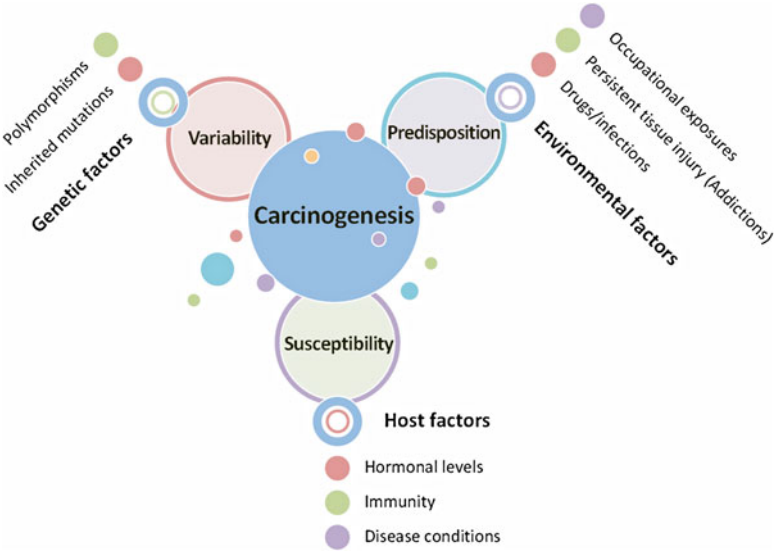

Fig. 2.1 Key contributing factors of carcinogenesis

microenvironment as compared to normal cells. This discriminatory microenvironment and altered pathophysiological signaling pathways have been classically used in the recent drug discovery approaches and set the genesis of molecularly targeted therapies.

Unfortunately, most of the anticancer drugs till time target the DNA or other biologicals actively involved in cell division and thereby control the rapidly diving cancer cells. However, in the course of that, the normal host tissues are also not spared and nonspecific generalized toxicity is noted which may be severely intense at times and may lead to either early termination of therapy or other secondary complications. The host tissues main targets include rapidly dividing lympho-hematopoietic cells, epithelial linings and other mucus secreting regions of gut, hair follicular regions, etc. These complexities lead to low chemotherapeutic index of the anticancer drugs. Secondly, rapid emergence of drug resistance also contributes majorly to the poor cancer chemotherapeutics [3]. Hence, there lies a strong need to develop selective anticancer therapeutics which would principally act to cancer cells without affecting the normal tissues. The materialization of the concept "magic bullets" seems to be mandatory considering the widespread prevalence of cancer.

Tumor targeting is defined as the improving the drug's chemotherapeutic index by (a) preferentially localizing its pharmacological activities at the site of action, (b) recognition and interaction with target cells, and (c) achieving cellular concentrations so as to exhibit therapeutic response [4]. Very often a variety of homing devices are being employed to direct the drug and/or carriers to the particular site of action. Mechanistically, these homing devices are the special molecular signatures that are expressed to a greater extent at the tumor tissues such as folic acid, etc. The principal need for tumor targeting is required due to limited accessibility of drugs to tumor tissues, requirement of high doses, intolerable cytotoxicity, development of multidrug resistance and nonspecific targeting [5]. However, although fascinating, the tumor targeting is often exposed to a variety of barriers mediated by peculiar tumor microenvironment.

2.2 Normal Vs Tumor Vasculature

Classically, there lies a prominent homeostasis among the proangiogenic and anti-angiogenic molecules in the normal tissues which are responsible for balanced organization of the blood vessels for meeting the metabolic demands. This system works in tandem with lymphatics for clearance of the cellular by-products. Carcinogenesis leads to imbalances in these systems leading to a variety of alterations. The chaos starts with alterations in the normal vasculature (abnormalities in the functional and structural aspects) leading to diminished nutrients supply and clearance of cellular waste products. Subsequently, compromised basal membrane, disorganized pericyte layer, downregulation of the adhesion molecules and endothelial linings contribute to high permeability of the tumor tissues.

Tumor vessels usually grow abnormally with unusual vessel diameter, lower vascular density and longer tortuous paths for diffusion of molecules. Further, fluctuant flow, poor plasma channels, and arteriolar-venous shunts for efficient RBCs supply are responsible for two types of hypoxic conditions, i.e., chronic due to compromised diffusion and acute due to lower exchanges, ultimately leading to heterogeneous tumor oxygenation supply [6]. Cumulatively, the principal barriers for the conventional chemotherapeutic agents to access the tumor tissues are malformed tumor vasculature which leads to altered vascular permeability, high interstitial pressure, extracellular acidosis, and hypoxia (due to high mitochondrial oxygen consumption) [7].

Secondly, the interstitial space is regarded as one of the important component for maintaining homeostasis in tissues. It is responsible for exchange of primary requirements of cells such as oxygen and nutrients along with clearance of waste products. In combination with hydrostatic pressure and colloidal osmotic pressure, the transcapillary flow maintains the hydraulic conductivity and plasma protein reflection coefficient among the cells and capillaries in the adjacent to the tissues (Fig. 2.2) [8]. Usually, this transcapillary pressure is slightly higher in capillary bed in the order of 1–3 mmHg so as to maintain the flow of solutes and water from capillaries to cells via interstitial spaces. However, in case of tumors interstitial pressure shoots up to 100 mmHg owing to three principal reasons. These include (a) compromised functionalities of the blood vessel and lymphatics, (b) osmotic pressure generated by drainage of solutes from tissues, and (c) high contractile characteristics of tumor tissues [7]. Interestingly, these are essential targets in the current drug discovery strategies for molecularly targeted therapies.

Thirdly, the pH of the tumor microenvironment is usually dropped relatively towards acidic, as evident from the direct measurement by placing sensitive electrodes into the solid tumors [9]. Classically, it has been widely accepted that hydrolysis of ATP via energy deficient pathways and anaerobic conditions leads to formation of acidic lactate moieties within tumor tissues [10]. Warburg studied the greater production of lactate within the tumor tissues as compared to that of normal tissues and was attributed to the respiratory impairment; however, exact biologics of reduction in tumor pH is not yet identified [11]. The recent advances using genetically modified tumor models revealed that existence of non-lactate mediated acidic microenvironments [12]. It further identified that there exists balance among the intracellular and extracellular pH mediated by proton pumps, which actually regulate the overall tumor pH [13]. Additionally, the uncleared cellular waste products also drastically contribute to the acidic tumor microenvironments [14].

2.3 Barriers to Tumor Targeting

The principal barriers associated with tumor targeting comprise peculiar tumor vasculature which principally comprises heterogeneous blood flow and vascular resistance [15]. In the purview of unregulated growth of tumor vasculature and there

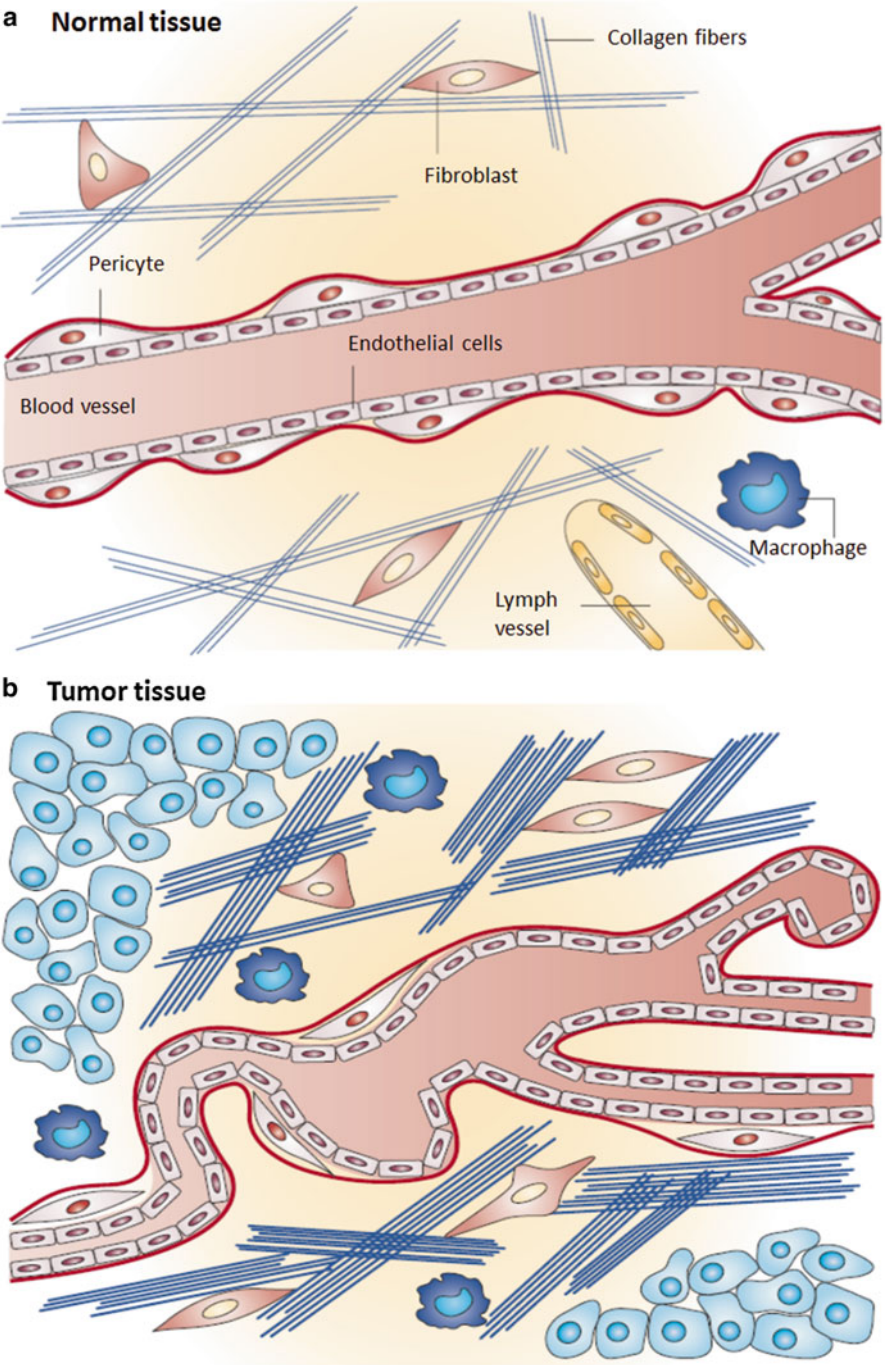

Fig. 2.2 Structural differences between normal and tumor tissues that affect interstitial fluid pressure. (**a**) Normal tissues contain linear blood vessels lined by a smooth layer of endothelial cells with pericytes maintaining the integrity of the vessel on its outside. The extracellular matrix

occurs nonuniform distribution of blood vessels across the tumor leading to patches of very high blood supply to almost negligible supply. This heterogeneity leads to uneven distribution of administered therapeutics often leading to poor therapeutic response. Such altered distribution also usually ends up in partial exposure of drug to the cells, thereby drastically increasing the multiple drug resistance with the tumor cells. Along with these, achievement of therapeutic responses of drug with cancer cells is further challenged by overexpression of efflux transporters, often referred to as ATP binding cassette (ABC) transporters such as P-glycoprotein, multidrug resistance proteins (MRP-1, -2), etc. Most of the anticancer drugs are substrates of such efflux transporters. This piece of information has been exhaustively reviewed by our group previously and is already available in scientific domain hence kept out of scope of this chapter [16]. Subsequently, other factors such as diffusional barrier due to high intercapillary distance, cell density, and extracellular matrix components also pose potential barrier to tumor delivery of therapeutics.

2.4 Conventional Strategies for Tumor Targeting

The principal goals of the targeted drug delivery system is aimed at protection of the drug in concern to the site of action from the metabolic degradation/inactivation during transit, particularity for specific target devoid of any nonspecific interactions with the host tissues and penetration of relevant concentrations of drug within the tumor tissues for therapeutic responses.

In this regard, selective accumulation of the drug at preferred site is also majorly affected by its physicochemical properties. Most of the anticancer drugs fall in the category II/IV of Biopharmaceutical Classification Systems (BCS), thereby posing pharmaceutical problems while water soluble drugs pose problems related to permeability across various biological barriers [16]. Classically to address these concerns, three major approaches could be employed which include (a) subtle structural modifications for improving the physicochemical properties in accordance with structure–activity relationships (SAR), (b) conjugating homing ligands for predetermined bio-distribution patterns, and (c) involvement of carrier based approaches

Fig. 2.2 (continued) consists of a loose network of collagen and other fibers, and contains a few fibroblasts and macrophages. Lymph vessels are also present in normal tissues. (**b**) Tumor tissues contain defective blood vessels that are leaky and irregularly shaped, with many sac-like formations, dead-ends and highly activated endothelia. Blood flow is therefore inefficient. These blood vessels are also covered by fewer pericytes than in normal tissues, resulting in decreased vessel stability. Furthermore, many tumors lack lymph vessels, so interstitial fluid and soluble proteins are inefficiently removed. The extracellular matrix of tumors contains a much denser network of collagen fibers, which are thicker than in normal tissues. Therefore, the tumor tissue is more rigid than normal loose connective tissue. Tumors also contain an increased number of fibroblasts, which bind to the collagen fibers in an integrin-dependent manner and exert an increased tension between the fibers, as well as an increased number of macrophages and other inflammatory cells; these cells release cytokines and growth factors that act on cells of blood vessels and stroma fibroblasts to increase interstitial fluid pressure. Reproduced from ref. [8]

for targeting the therapeutics at site of action [17]. Alternatively, targeting could be categorized as either passive or active depending upon the approach employed.

2.4.1 Passive Targeting

The natural biodistribution pattern of the drug delivery carrier is exploited for its preferential localization in the vicinity of the tumors such as enhanced permeation and retention effects, phagocytosis of particulate carrier by mononuclear phagocytosis systems (MPS) and preferential localization in the organs of reticuloendothelial system (RES). In addition, other typical properties of the tumor microenvironment such as low extracellular pH, relative micro-acidosis, mild hyperthermia, etc. could also be employed for availing passive targeting of therapeutics. However, the targeting potential of such a strategy is relatively low and often associated with partial nonspecific localization of therapeutics in the normal tissues which needs to be considered while employing such therapies.

2.4.1.1 Enhanced Permeation and Retention (EPR) Effect

The EPR effect was first noted three decades ago for the preferential localization of protein macromolecules in the vicinity of the tumor and since then it has been widely explored for the alteration in biodistribution patterns of most of the colloidal drug delivery systems such as liposomes, polymeric nanoparticles, polymer drug conjugates, etc. However, with the advent of the increased research in this field, EPR effect has been regarded as blanket terminology for increased efficacy of any cancer therapeutics. Aggressive studies in this direction suggested EPR effect as complex association of various processes such as angiogenesis, vascular permeability, hemodynamic regulation, genetic heterogeneities among tumors, lymphangiogenesis, and heterogeneous tumor microenvironment [18].

Classically, the cell proliferation leads to formation of solid mass and upon reaching a specific size, cells in the interior starts getting deprived of the nutrients which leads to cell death and release of growth mediators signaling the development of the blood vessels within tumor. However, the formed blood vessels are often leaky owing to absence of basal membrane leading to fenestrations within the size of 200–2,000 nm [19]. The presence of fenestrations results in poor resistance to the extravasation of macromolecules to the tumor microenvironment and contributes to the enhanced permeation part of EPR. Simultaneously, it has also been found that tumor mass is associated with nonuniform lymphatic drainage and experience a huge physical stress owing to rapid growth in the dimensions of the tumor mass [20]. This leads to the severe compromise in the drainage functionality of the vessels and contributes to the retention part of EPR effect [21].

Principally, the EPR effect is mediated by extravasation of the macromolecules from the blood vessels followed by the subsequent movement in the tumor

microenvironment via diffusion and convection. The principal factors affecting EPR effect includes vessel architecture, interstitial fluid composition, extracellular matrix composition, phagocyte infiltration, presence of necrotic domains, factors pertaining to the colloidal carriers such as blood circulation time, particle size, particle shape, surface charge, and surface functionalization, if any, (e.g., stealth characteristics by PEGylation). Exhaustive review on the factors influencing EPR effect and mobility of the colloidal carriers has been recently compiled and hence kept out of the scope of this chapter [18].

2.4.1.2 Surface Engineering of Colloidal Carriers for Stealth Characteristics

The colloidal carriers by virtue of their inherent properties are rapidly taken up by the mononuclear phagocyte system (MPS) via process of opsonization. However, drastic reduction in RES uptake and significant appreciation in the EPR effect of the colloidal carriers can be achieved by surface engineering [22]. Usually, the opsonins interact with the colloidal carriers via forces such as van der Waal's forces, weak electrostatic forces, ionic forces, and hydrophobic/hydrophilic forces. In purview of this, hydrophobic and charged particles are rapidly processed by RES and significant prolongation in the circulation half-life can be achieved by surface functionalizing PEG chains forming "stealth" systems [23].

A variety of natural materials such as dextrans, pullulans, gangliosides, etc. have been employed for proving stealth characteristics to the colloidal carriers. Of note, gangliosides represent the class of glycosphingolipids containing sialic acid and are regarded as integral component of plasma membrane, particularly red blood cells. The derivatives GM1 and GM type III have been exclusively explored for their potential in imparting stealth characteristics and appreciation in circulation half-life, while reduction in uptake by spleen and liver has been noted at numerous instances [24, 25]. Mechanistically, the stealth characteristics are imparted by steric barrier, shielding of anionic charge, and binding with dysopsonins [26].

The synthetic alternative of the natural polymers for imparting "stealthness" includes polyethylene glycol and their derivatives which have been widely explored and are often associated with numerous advantages such as simple anchoring process, biocompatibility, high solubility, stability, ease of availability at relatively inexpensive cost, flexibility in functionalization, etc. [22]. Although fascinating, the PEGylation of colloidal carriers is also associated with a variety of drawbacks such as significantly higher hydrophilicity hinders the efficient hydration of polar head groups of phospholipids leading to poor stability and problems of drug leaching [27], often necessitating higher levels of cholesterol to prevent aggregation and phase separation [28]. Secondly, there have been some instances of immunogenicity by PEGylated colloidal carriers resulting in hypersensitivity reactions [29]. The activation of complement system and induction of anti-PEG antibodies (IgM) has been observed to rapidly clear off the circulating PEGylated colloidal carrier by a mechanism called ABC phenomenon and is highly detrimental on appreciation in

bioavailability, passive targeting, and ultimately efficacy of the system, per se [30]. Further the long term safety of the PEGs is also scarcely established particularly the biological fate. In purview of this, physiological metabolism of PEGs (<400 Da) includes alcohol dehydrogenase mediated oxidation leading to formation of toxic diacid and hydroxyl acid metabolites [31]. On the other hand, the renal clearance cutoff for PEGs is 30–50 kDa, further narrowing the limits for its clinical use [32]. Hence, a series of alternative synthetic derivatives are currently being explored which include vinyl based lipopolymers, polyoxazolines based lipopolymers, polyamino based lipopolymers, zwitterionic lipopolymers, etc.

2.4.2 Active Targeting

Active targeting refers to the attachment of marker component to the colloidal carrier system which is specifically recognized by the target in concern may it be either from organelle or organ. Usually molecular targets are employed such as overexpression of surface receptors on tumor cells for site specific delivery of therapeutics such as dietary ligands (carbohydrate based, folate, etc.), monoclonal antibodies and their fragments, non-antibody ligands (peptidic ligands), etc. The active targeting could be divided into various levels depending upon extent of penetration, i.e., organ level, cellular level, and subcellular level. However, independent of the target location, the preliminary characteristic of the targeting ligand is its specificity which should be neither upregulated nor downregulated upon exposure to physiological conditions [33]. Concomitantly, the binding affinity of the targeting ligands should also remain unchanged which indirectly is affected by the binding site barrier leading to altered tumor penetration. At times very high binding affinities are required considering the higher mobility of the colloidal carrier systems in the physiological conditions.

2.4.2.1 Albumin Based Targeting

Albumin plays a critical role in maintaining the homeostasis by mobilizing key endogenous hydrophobic molecules. It specially binds via non-covalent interactions and executes the transport of molecules in concern by transcytosis across the endothelial cells into interstitial space. Paclitaxel bound albumin nanoparticle represents the classical example for establishing the potential of albumin based delivery of anticancer drugs [34]. Mechanistically, it binds to the gp60 receptor present at the cell surface and leads to the activation of the caveolin-1 mediated transcytosis which also unintentionally transports some of the unbound plasma constituents [35, 36]. Concomitantly, tumor cells also secrete albumin binding proteins, SPARC, also referred to as BM-40, which are acidic in nature and rich in cysteine, binding to the albumin tagged colloidal carrier systems. Such a system could fruitfully be exploited

for targeting the therapeutics to the brain via adsorptive mediated transcytosis. Cationized albumin significantly increased the uptake of β endorphin in isolated brain endothelial cells as compared to its native form [37]. Furthermore, ~4-fold increase in the cellular uptake of albumin bound paclitaxel by endothelial cells has been noted as compared to the clinical formulation, Taxol® which was completely inhibited upon coadministration with β cyclodextrin, the known inhibitor of gp60 suggestive of the active transport as predominant uptake mechanism for albumin based nanoparticles [38]. The principal advantages associated with albumin based targeting include superior stability over a wide range of pH (4–9) and temperature (10–60 °C), biodegradation, non-immunogenic, and nontoxic. A striking advantage includes its additional cryoprotectant effect which makes the lyophilization of formulation in concern quite easier than other systems in race.

2.4.2.2 Vitamin Based Targeting

The vitamins employed for targeting potential includes folate, vitamin B_{12}, thiamine, and biotin [39]. The principal advantages associated with vitamins, particularly folic acid, includes stability over shelf and physiological conditions, relatively inexpensive, nontoxic, non-immunogenic, endogenous homing ligand, wide flexibility for diverse chemical reactions, and relatively higher overexpression of folate receptors on most of the cancers [40]. It has been noted that folate functionalized colloidal carrier systems are preferably absorbed by receptor mediated endocytosis. Folate functionalized nanoparticles have been widely explored by numerous research groups including ours for its potential in preferentially localizing the therapeutics in the vicinity of the tumor tissues. Our group has developed methotrexate loaded folate functionalized albumin nanoparticles for significantly improving its antitumor efficacy and reducing the toxic side effects by virtue of altered biodistribution pattern to target tumor tissues as evident by pharmacoscintigraphic evaluation [41]. In a separate set of experiments functional magnetite nanoparticles have also been explored for active targeting potential which were found to selectively target and induce apoptosis in folate receptor overexpressing cancer cells, thereby imparting significantly higher anticancer properties as compared to parent drug [42].

Furthermore, folic acid functionalized carbon nanotubes have also been explored to a greater extent to assess its potential for cancer theranostic applications which comprised fluorochrome (Alexa Fluor 488/647), radionuclide (Technitium-^{99}m), tumor-targeting module (folic acid), and anticancer agent (methotrexate) [43]. The developed system exhibited significantly higher internalization within lung cancer cell lines (A549) and breast cancer cell lines (MCF-7) as evident by the lysosomal trafficking and resulting in higher anticancer activity. Subsequently in vivo experiments revealed ~19-fold increase in the tumor localization for the targeted formulation as compared to free drug. Table 2.1 reveals the representative list of formulation approaches employed for improving the tumor delivery of therapeutics using folate as targeting ligand.

Table 2.1 Folate conjugated nanoparticles for improved tumor delivery of therapeutics

Delivery system	Drug	Outcomes	Ref.
Magnetic multi-walled carbon nanotubes	Doxorubicin	Efficient uptake by U87 cells and higher intracellular release of DOX	[44]
HPMA copolymer conjugate	Doxorubicin	Higher apoptosis and greater tumor spheroid inhibition against Hela cells	[45]
High-density lipoprotein nanoparticles	–	Enhanced selectively towards ovarian cancer cells	[46]
Superparamagnetic iron oxide (Fe_3O_4)	Doxorubicin	~2.5-fold higher than that for the non-targeting group.	[47]
Folate-tagged liposomes	Ricin	Significant increase in the cytotoxicity up to 557.7-fold was demonstrated by monensin intercalated folate liposomes	[48]
pH responsive polymeric nanoparticle	Doxorubicin	Increased targeting efficiency of polymeric nanoparticles, resulted in enhanced cellular uptake by 100-fold	[49]
PLGA nanocapsules	Quercetin	Folate modified PLGA nanocapsules showed selective uptake and cytotoxicity to folate expressing Hela cells	[50]
Poly(L-γ-glutamyl glutamine) (PGG) nanoparticle	Docetaxel	Folate targeted PGG nanoparticle system was found to be highly effective against tumor cells and successfully localized in the tumor site	[51]
Polyhedral oligomeric silsesquioxane-F68 hybrid vesicles	Doxorubicin	Significantly enhanced the uptake in Hela and HOS cells	[52]
Polymersome	Doxorubicin	Higher anti-glioma effect compared to the treatments with free doxorubicin	[53]

2.4.2.3 Transferrin Based Targeting

Transferrin receptors are also exclusively overexpressed in most of major types of tumors including lung, lymphomas and breast cancers in the order of ~10-fold [54]. The important feature of employing transferrin as targeting ligand is its capability for enabling the transcytosis across blood brain barrier [55]. Sahoo et al. exhaustively explored the potential of transferrin conjugated paclitaxel loaded nanoparticles for variety types of cancer including breast cancer and prostate cancer [39]. Significantly higher levels of paclitaxel were noted in the case of transferrin conjugated nanoparticles during cell uptake studies as compared to that of free drug and non-targeted formulation counterparts [56]. Furthermore, in separate set of experiments, transferrin conjugated nanoparticles revealed about threefold higher uptake in PC-3 cell lines and concomitantly significant increase in therapeutic efficacy was noted for the developed formulation in in vivo murine model as compared to that of free drug and non-targeted formulations [57]. Table 2.2 represents the representative list of transferrin conjugated nanoparticles employed for improving the tumor delivery of therapeutics.

Table 2.2 Transferrin conjugated nanoparticles for improved tumor delivery of therapeutics

Delivery system	Drug	Outcomes	Ref.
Mesoporous silica nanoparticles	Camptothecin	Enhanced uptake by Panc-1 cancer cells and toxicity of cancer cells as compared to normal cells	[58]
TRAIL (TNF-related apoptosis-inducing ligand) nanoparticles	–	5.2-fold higher tumor accumulation	[59]
Liposomes	Doxorubicin	Significant improvement in survival time	[60]
Pegylated nanoscaled graphene oxide (GO)	Doxorubicin	Enhanced intracellular delivery, efficiency and stronger cytotoxicity	[61]
Lipoplex	Cytosine deaminase	Significant tumor reduction and enhanced apoptosis	[62]
DQAsomes	Paclitaxel	Higher uptake and tumor cytotoxicity	[63]
Polymeric micelles	Curcumin and Paclitaxel	Improved cytotoxic effect against the SK-OV-3 cells	[64]
Polymeric nanoparticles	si-RNA	Marked tumor accumulation	[65]
Selenium nanoparticles	Doxorubicin	Significantly enhanced cellular uptake	[66]

2.4.2.4 Lectin Based Targeting

Lectins represent a class of cyto-adhesive targeting ligands which is moderately recognized by glycans on the glycosylated cell surface proteins and lipids. Most of the cell surface expresses peculiar glycan arrays which can be sensed differentially and hence this could be a viable strategy as regards targeting perspectives [67]. The targeting potential of lectins has been explored in a wide field of applications including gastrointestinal targeting, nasal delivery, pulmonary delivery, buccal cavity, ocular drug delivery, and brain delivery. Of note, targeting of liver targeting has also been quite possible using lectins for delivering drugs and genes. The asialoglycoprotein receptors are specifically overexpressed on liver which recognizes either β-galactose or *N*-acetyl galactosamine residues [68]. Interestingly, this approach could also be employed using polymer drug conjugates wherein drug and galactose residues can be covalently linked to polymer backbone [69]. On similar line of action, asialofetuin tagged liposomes have also been explored to improve the hepatic delivery of hydrophilic molecules [70].

2.4.2.5 Peptide Based Targeting

Peptide based tumor targeting strategy is considered as most promising because relatively higher stability and smaller size of tumor specific peptides. The peptides

employed for tumor targeting could be either monomeric, homodimeric, heterodimeric oligomeric or tetrameric in nature. Cyclic RGD peptide anchored liposomes were previously prepared preferentially targeting anticancer drug 5-fluoro uracil to tumor vasculature. In vitro endothelial cell uptake studies revealed significantly higher uptake of RGD labeled liposomes as compared to non-targeted counterparts leading efficient prevention of spontaneous lung metastasis and angiogenesis[71]. The tumor specific peptides could be broadly categorized into two categories, one targeting tumor cell surface while other targeting tumor vasculature. The cell surface targets could be either lymphomas, myelomas, neuroblastomas, breast cancer, head cancer, neck cancer, prostrate cancer, endothelial cells, or human laryngeal carcinomas whereas the tumor vasculature targets could be $\alpha_v\beta_3$, $\alpha_v\beta_5$, aminopeptidases, proteoglycans, gelatinases, and vascular endothelial growth factors [72].

2.4.3 Physical Targeting

A variety of physical approaches have also been explored for their potential to preferentially localize anticancer medicaments in the vicinity of tumors. The physical stimuli for drug targeting may either be endogenous such as pH, temperature, redox potentials, etc., or be exogenous, i.e., employment of external forces such as magnetic, ultrasound, etc. [73]. As discussed earlier, the tumor microenvironment is slightly acidic and exhibits mild hyperthermia which could be specifically exploited as a stimulus for physical targeting. Stimuli responsive colloidal systems have been designed and developed that tend to degrade at acidic pH and/or elevated temperatures. On the other hand, magnet assisted tumor targeting approaches have also widely been explored considering its immense potential. In this particular system, the drug in concern is immobilized on ferromagnetic colloidal carriers and allowed to circulate in body. The external magnetic field is applied at the site of action which localizes the circulating carriers leading to exceptional tumor levels of drugs. Similarly, the circulating colloidal carrier may be accumulated at the desired site of action using ultrasound energy. Significantly higher tumor levels of doxorubicin were noted from polymeric micelles upon imparting external ultrasound as compared to that of free drug counterpart [74]. The driving force for preferential localization herein is the destabilization of colloidal carrier upon exposure of high energy external force.

2.5 Recent Advances in Tumor Targeting Approaches

2.5.1 Molecular Targeted Therapies

Persistent tissue injuries to the cells and/or factors generally tend to dysregulate the well organized signaling systems of cell cycle and ultimately leading to tumorigenesis. Further, understanding of the fact that either a particular site of molecule or

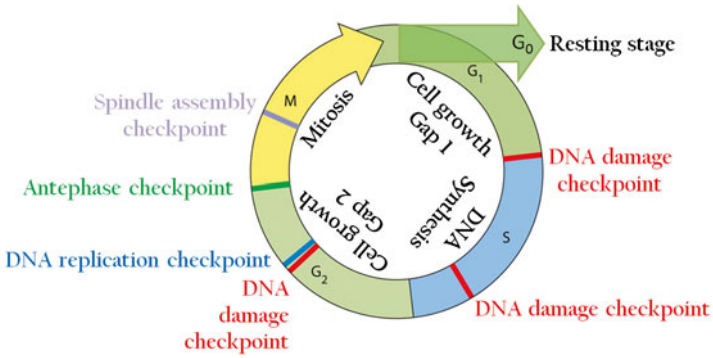

Fig. 2.3 Cell cycle checkpoint pathways

whole molecule itself can play very diverse role in the normal cells and cancer cells, makes the things quite complicated yet interesting. In this regard, molecular targeted therapies are sought and principally include agents which act on aberrant functions and expression of cell cycle involved in the pathophysiology of cancer such as interference with the pathways exclusively expressed in tumor cells. The cell cycle comprises four phases, viz., G_1, S, G_2, and M phase. Depending upon the cell signaling, the cells in G1 phase determines whether to proceed with S phase, apoptosis, or G_0 phase. Upon entering the S phase, DNA synthesis takes place which is followed by G_2 phase ultimately enabling the cell to enter M phase where cell division occurs and cell cycle continues. Concomitantly, the cell cycle processes are also regulated by a variety of kinases referred to as cyclin dependent kinases (CDKs) [75]. A series of CDKs and CDKs inhibitors have been known which are constantly employed for ensuring correct cell division processes [76]. Some of these CDKs also monitor checkpoints that cover DNA damage, antephase, and spindle assembly (Fig. 2.3).

In contrast, tumorigenesis involves multiple complex set of conditions wherein the genetic aberrations and dysregulation of cell cycle occur. Apart from downregulation of tumor suppressor genes such as TP53, BRCA1, BRCA2, alterations in the cell cycle also contribute equally to tumor progression. A variety of mediators have been known which actually bridge the gap between dysregulation of cell cycle and genomic instability such as telomere crisis [77]. Secondly, downregulation of retinoblastoma tumor suppressor protein (pRb2) is regarded as the hallmark of tumor cells and mediates by overcoming the S-phase checkpoint and is referred to as CDK/$p16^{INK4A}$/pRb pathway. Thirdly, p53/HDM2/$p14^{ARF}$ pathway is also considered one of the major cell cycle surveillance pathway operated by HDM2 gene amplification or p53 gene alterations [75]. It affects G1 checkpoint and is sensitive to a variety of stress signals such as DNA damage, hypoxia, etc. Of note, E3 ubiquitin ligase is the key enzyme responsible for p53 ubiquitylation and proteasome inhibition resulting in transcriptional changes and completes negative feedback loop. Inhibition of the CDKs is an important target and is explored to a greater extent. These strategic inhibitors could be designed to compete via inhibition of the ATP binding sites or upregulation of the native CDK inhibitors. Among various

cyclins known till date, cyclin D and E are often found to be overexpressed in a variety of malignancies [78, 79].

In the purview of molecular targeted therapies, the available bioactives could be crudely categorized among three broad generations, viz., first generation comprising ones that act predominantly via DNA damage, synthesis and/or other linked processes such as tubulins, second generation comprising agents that target cancer growth signaling mechanisms such as kinases, etc., and third generation, which is actively updated and is regarded as most recent and under development, comprising agents which act on cellular pathways indirectly related to cancer growth such as chromatin modifiers, protein chaperones, proteasome inhibitors, etc. (Fig. 2.4) [80].

The classical problems associated with first generation anticancer agents include genesis of secondary cancer such as multiple neoplasms in the pediatric patients survived with childhood cancers [81], long terms survivors of patients suffering from testicular carcinomas [82], etc. Furthermore, it also severely affects rapidly proliferating normal cells such as hair follicles, cells of hematopoietic system, gastrointestinal tract lining, and so on. In addition, detrimental effects on post mitotic tissues such as cardiac muscles and that of peripheral nervous systems have also been observed [83, 84].

Second generation molecularly targeted anticancer therapeutics, to some extent addressed the complications associated with classical drugs. Oncogene addiction and non-oncogene addiction targets have been identified which are either direct gene alterations or indirect alterations, respectively [85]. These alterations could be either due to gain- or loss-of-function mutations, amplification and/or overexpression of oncogenes such as MYC, Rb, p53, etc. Recently, it was identified that close to 20 % of kinases play critical role in tumorigenesis[86]. In this regard, Gleevac (imatinib), a potent inhibitor of tyrosine kinase, was the first product approved clinically for chronic myeloid leukemia [87]. Subsequently, a series of drug compounds were approved which includes Lapatinib (HER2 and epidermal growth factor receptor), vemurafenib (B-Raf), vismodegib (Hedgehog signalling pathway), ruxolitinib (Janus kinases), gefitinib (epidermal growth factor receptor), Sunitinib, sorafenib, and pazopanib (multiple tyrosine kinases), and tivatinib (hepatocyte growth factor receptor) [80]. In addition, the monoclonal antibodies, discussed in latter part of this chapter are also considered as second generation therapeutics. The other miscellaneous agents included under this category are non-oncogene addition targets such as checkpoint kinases [88], mTOR[89], etc. Although these agents act predominantly at oncoprotein targets and are less prone to toxic side effects, acquired resistance has been observed quite often with most of the drugs.

Third generation of molecular targeted therapies is further in move considering the complications associated with available drugs which focusses mainly on DNA synthesis, replication, repair, and cell division. These agents include:

2.5.1.1 Agents Acting on Protein Folding and Proteotoxic Stresses

Considering the typical microenvironment in the vicinity of tumor, cancer cells constantly experience a variety of stresses, especially permanent proteotoxic stress

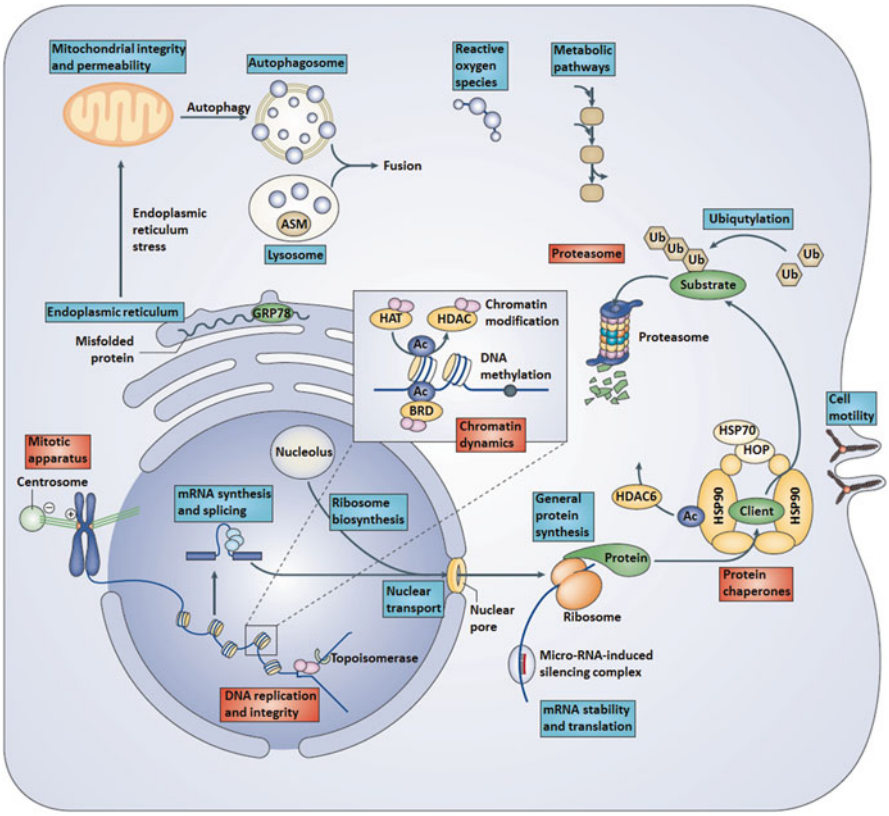

Fig. 2.4 Cellular multicomponent machineries as current and future targets for anticancer drugs. Current targets (shown in *red boxes*) include: DNA replication and integrity; the mitotic apparatus; chromatin; protein chaperones; and the protein degradation apparatus (the proteasome). Drugs that target DNA replication and integrity act via the following mechanisms: by crosslinking nucleobases in DNA and blocking DNA replication; by inhibiting DNA repair; by inserting planar polyaromatic molecules between DNA base pairs and stabilizing the DNA–intercalator–topoisomerase II ternary complex; by interfering with the polymerization of DNA (e.g., via the incorporation of nucleoside analogues); and by inhibiting nucleotide synthesis, typically using antagonists of ribonucleotide reductases or thymidine synthetase. Drugs that target the mitotic apparatus act by binding to the inner portion of microtubules (the "–" end; e.g., taxanes and epothilones), presumably leading to stabilization and enhanced rigidity of the spindle. Vinca alkaloids bind to the "+" end of microtubules—that is, the end that usually elongates the microtubule by adding subunits of α- and β-tubulin—thereby destabilizing the microtubule. Chromatin modification can be targeted by drugs that act on cellular enzyme complexes such as histone deacetylases (HDACs), bromodomain-containing proteins (BRDs) and DNA methyltransferases. Protein chaperones assist in refolding mutated or stress-misfolded proteins. Complexes consist of the heat shock proteins HSP90 and HSP70 (both of which are ATPases), as well as HSP90 organizing protein (HOP; also known as STIP1), multiple co-chaperones, adaptor proteins, the ubiquitin E3 ligase CHIP (carboxy terminus of HSP70 interacting protein) and the associated HDAC6 (a positive regulator and a cytoplasmic deacetylase that keeps HSP90 deacetylated and active). Drugs can inhibit HSP90, HSP70, or HDAC6. Drugs can inhibit different protease activities—e.g., chymotrypsin-like activity, trypsin-like activity, and/or caspase-like activity—within the 26S proteasome to disrupt the protein degradation apparatus. The ubiquitylation machinery and ubiquitin retrieval can also be manipulated by small molecules, providing additional opportunities for interfering with proteasomal degradation. Future targets for third-wave anticancer drugs are illustrated in *blue boxes*. *ASM* Acid sphingomyelinase, *GRP78* 78 kDa glucose-regulated protein. Reproduced from [80]

which is generally caused by the misfolding and aggregation of proteins. The latter effect is predominantly observed in cancer cells owing to molecular crowding of the cellular milieu [90]. Usually such stresses are counterfeited by a group of molecules inclusive of chaperones and protein remodeling factors. Many of these are responsive to heat and hence referred to as heat shock proteins (HSPs). HSPs interact with their client protein with the help of co-chaperones; however, under extremities of cellular stresses owing to oncogene alterations, chaperone pool vanishes quickly and chaos originates. In complementarily reactive oxygen species further exaggerates the situation and lead to even higher proteotoxic stress [91]. Taking HSP90 as potential target, a variety of therapeutics have been designed for molecular targeting of cancer which includes 17-allylamino-17-demethoxygeldanamycin (17 AAG), ganetespib, STA-9090, IPI-493, retaspimycin, tanespimycin, geldanamycin, radicicol, AT-13387, NPV-AUY922, KW-2478, BIIB-021, MPC-3100, NVP-HSP990, PU-H71, etc. [92] (Table 2.3).

2.5.1.2 Proteasome Inhibitors

Ubiquitin-proteasome system is yet another approach to address the proteotoxic stress covering almost 90 % of the total protein clients [93]. Mechanistically, it follows two steps essentially comprising ubiquitin conjugation mediated by a series of enzymes such as E1 (Ub-activating), E2 (Ub conjugating), and E3 (Ub ligating) to yield Lys48 linked proteins which are then processed for proteasomal degradation via 26S proteasome complex (Fig. 2.5). Herein tumor cells, the proteasome functions and its need is always unmet and hence pose a potential target in anticancer therapy. Concomitantly, the PSMC2 gene alterations further adds complexity to the overall situation. The classical proteasome inhibitors were designed to interact with proteasomal components such as 20S core subunit (Bortezomib, carfilzomib, etc.) [94], interference in the deconjugation of ubiquitin and substrates [95], inhibition of ubiquitin specific peptidase-14 [96], allosteric inhibition of E2 enzyme [97], neddylation[98], etc.

2.5.1.3 Targeting Chromatin Modifications

Chromatin is crudely regulated by three categories of enzyme systems, viz., epigenetic writers, epigenetic erasers, and epigenetic readers [99]. The first category includes enzymes which are responsible for adding chemical moieties to histones or DNA such as acetyltransferases, methyltransferases, etc. The second set of enzymes is primarily responsible for deletion of groups and includes deacetylases, demethylases, etc. The third group comprises the protein modules that are responsible for chromatin binding leading to either upregulation or downregulation of transcription processes, e.g., H3K4me3/2-specific histone demethylase. H3K4me3/2 is a ligand specific for plant homeodomain finger (PHD), zinc finger-like domain, with Cys4-His-Cys3 signature motif [100]. The principal chromatin modification targets

Table 2.3 List of Hsp 70 target based drug delivery system for cancer immunotherapy

Hsp 70 inhibitor	Source	Remarks
Geldanamycin (GA)	Natural benzoquinone ansamycin antibiotic	Compete at the ATP-binding site to induce the degradation of Hsp90 via the proteasome machinery of ubiquitin ligase
Radicicol (RD)	Natural macrocyclic lactone antibiotic	Inhibitory effect against tyrosine kinases countered by reducing agents such as dithiothreitol
17-DMAG	Derivatives of GA	Increased water solubility and better oral bioavailability
IPI-504	Hydroquinone derivative of 17-AAG	IPI-504 shows higher water solubility and also high mortality rate
IPI-493 (17-AG)	Metabolite of 17-AAG	Longer circulation time
KF25706	Oxime derivatives of RD	Stable in the presence of dithiothreitol (DTT)
Herbimycin A (HA)	Benzoquinoid ansamycin antibiotic	Tyrosine kinase inhibitor and exhibits severe hepatotoxicity
KW-2478	Non-ansamycin resorcinol derivatives	KW-2478 caused degradation of FGFR3 as well as Hsp90 proteins, i.e., IGF-igβ and c-Raf-1, which resulted in cleavages of PARP and activation of intrinsic apoptotic pathway
NVP-AUY922	Resorcinol derivatives	Evaluated in Phase I/II clinical trial for NSCLC, breast cancer, colorectal cancer, and advanced gastric cancer and visual toxicity, i.e., night blindness and blurred vision, was reported
HSP990	Resorcinol derivatives	Acts via proteasomal degradation of oncogenic client proteins
AT13387	Resorcinol derivatives	Evaluated in Phase II clinical trial for gastrointestinal stromal tumor in combination with imatinib
Gatenespib (STA-9090)	Resorcinol derivatives	Evaluated in multiple clinical trials for both advanced solid
		Tumors (NSCLC, colorectal, stomach, ocular melanoma, pancreas, prostate, breast) and hematological malignancies
BIIB-021 (CNF2024)	Purine-scaffold based rational drug	Evaluated in phase I clinical trials for advanced solid tumor, B-cell chronic lymphocytic leukemia
SNX-5422/ PF-04929113	Pyrazole containing scaffolds	SNX-5422/PF-04929113 is water-soluble prodrug of SNX-2112/PF-04928473 and is discontinued in Phase I study due to ocular toxicity

include methylation of DNA and a variety of other histone modifications such as acetylation, ubiquitylation, phosphorylation, etc. Two drugs, 5 azacytidine and decitabine represent the pioneer drugs approved for myelodysplastic syndrome. Recent advances in this area of molecular targeted therapeutics include design of novel agents affecting chromatin modifications such as histone deacetylase (HDAC)

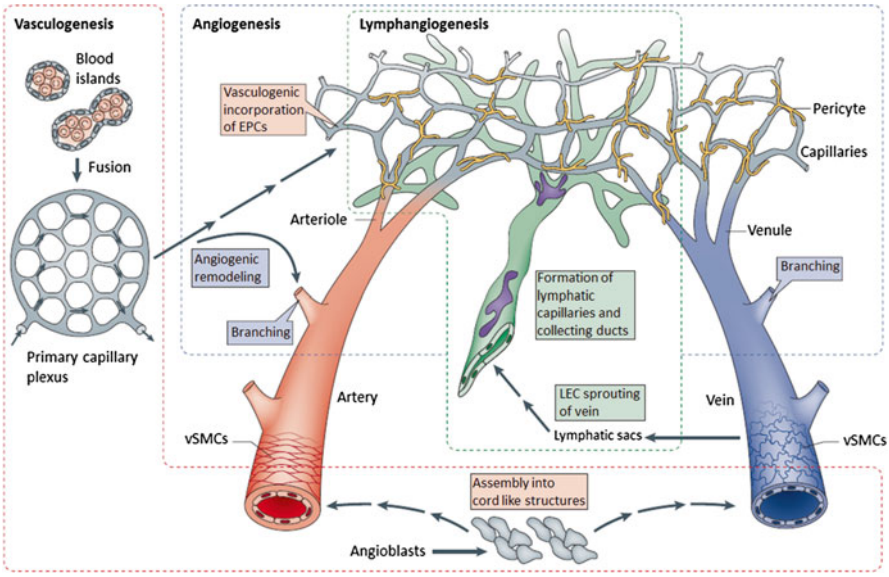

Fig. 2.5 Origin of endothelial cells and assembly of the vasculature. Mesodermal cells in the early embryo differentiate into endothelial precursor cells (EPCs, angioblasts) and form aggregates, known as blood islands (*left*). Fusion of blood islands leads to the vasculogenic formation of honeycomb-shaped primary capillary plexi in the yolk sac and embryo itself. Blood circulation is established and primary plexi are remodelled into a hierarchical network of arterioles and arteries (*red*), capillaries (*grey*), and venules and veins (*blue*). The dorsal aorta and cardinal vein are directly formed through the assembly of angioblasts. The vasculogenic incorporation of circulating EPCs into growing blood vessels may contribute to regenerative or pathological neovascularization in the adult. Vascular smooth-muscle cells (vSMCs) are associated with arteries and veins, whereas capillaries are covered by pericytes (*yellow*). The first lymphatic endothelial cells (LECs) sprout from the embryonic veins, then migrate and form lymphatic sacs. Further steps of lymphangiogenic growth involve sprouting, branching, proliferation, differentiation and remodeling processes. The recruitment of lymphangioblasts from the adjacent mesenchyme has been speculated to be a further source of LECs. Blind-ending lymphatic capillaries (*green*) feed into collecting vessels and ducts. These larger lymphatics are sparsely covered by SMCs (*purple*) and contain valves that prevent backflow. Reproduced from [108]

inhibitors, bromodomain and extra-terminal (BET) proteins downregulators, EZH2 inhibitors affecting histone methyltransferases, etc. [80].

2.5.2 *Tumor Angiogenesis*

In routine physiological conditions, the vascular network develops in three phases, viz., vasculogenesis, angiogenesis, and vascular remodeling. The first step comprises migration of angioblasts to the desired site followed by differential into endothelial cells and subsequent formation of initial vascular plexus [101]. The primary

vascular plexus then undergoes angiogenesis either sprouting or non-sprouting in the presence of various endothelial growth factors and is subjected to remodeling (Fig. 2.5) [102]. Classical long held view on the tumor angiogenesis was restricted to the fact that blood vessels in the tumor microenvironment grow only from the preexisting vessels. However, the recent advances in the field of vascular pharmacology revealed that vasculogenesis contributes majorly in tumor progression and share of endothelial cells derived from endothelial progenitor cells goes shoots beyond 40 % [103]. In purview of this, a variety of factors drives angiogenesis and includes vascular endothelial growth factors (VEGFs), fibroblast growth factors, angiopoeitins, netrins, semaphorins (class 3), SLIT proteins, JAG1, DLL4, ephrins, etc. The process of neovascularization is of utmost importance and starts as early as embryogenesis. However, angiogenesis has also been classically associated with a list of pathological conditions including cancer. Although the developmental and pathological angiogenesis operates on similar line of action, the major difference among both is that the latter remains unresolved and principally driven by pathological condition to address the unmet demands of nutrients and oxygen for cells in concern [104].

Crudely, angiogenesis is initiated in tumor upon reaching the size 1–2 mm and is typically coordinated by the hypoxic microenvironment of the tumor. Hypoxia inducible factors (HIFs), heterodimeric transcription factors, direct the expression of VEGF-A for angiogenic sequences [105]. Interestingly, since a series of physiological mediators are required for developing a fully functional vascular system, mere overexpression of VEGF renders aberrantly formed vessels that are often tortuous, fragile, pericyte deficient and leakier to raise the interstitial hypertension and often poor delivery of therapeutics to tumors [106]. Yet another complexity associated with tumor angiogenesis is *vasculogenic mimicry* which is actually a dedifferentiation program wherein the stem-like cells assist in formation of vascular system [107]. Cumulatively, these factors needs to be considered while employing and designing the antiangiogenesis based cancer chemotherapy.

Angiogenesis is considered as very dynamic process and is often regulated by a variety of indigenous angiogenic inducers and inhibitors. The former category includes principally VEGF-A, matrix metalloproteinases, fibroblast growth factors, placental growth factors, hepatocyte growth factors, etc., whereas the latter comprises thrombospondins, endostatin, angiostatin, and cytokines [109]. In the purview of antiangiogenic therapy, Fig. 2.6 depicts the possible targets for efficient management of tumor angiogenesis[103]. Classically, two approaches have been employed for targeting key regulators, i.e., VEGFs. Physiologically, VEGFs signaling system comprises five targeting ligands, VEGF-A to D and placental growth factor and three receptors, viz., VEGFR1-3 tyrosine kinases. The first approach comprises the use of antibodies for VEGF or its receptors and a series of drugs have already been approved clinically such as Bevacizumab (humanized variant, VEGF) and some others are under investigation such as VEGF-$\text{Trap}_{\text{R1R2}}$. However, the use of such antibodies is associated with side effects, pharmacoeconomic complications, etc. and hence second approach of VEGF receptor kinases inhibitor could also be sought for. Sorafenib was the pioneer candidate in this category to be

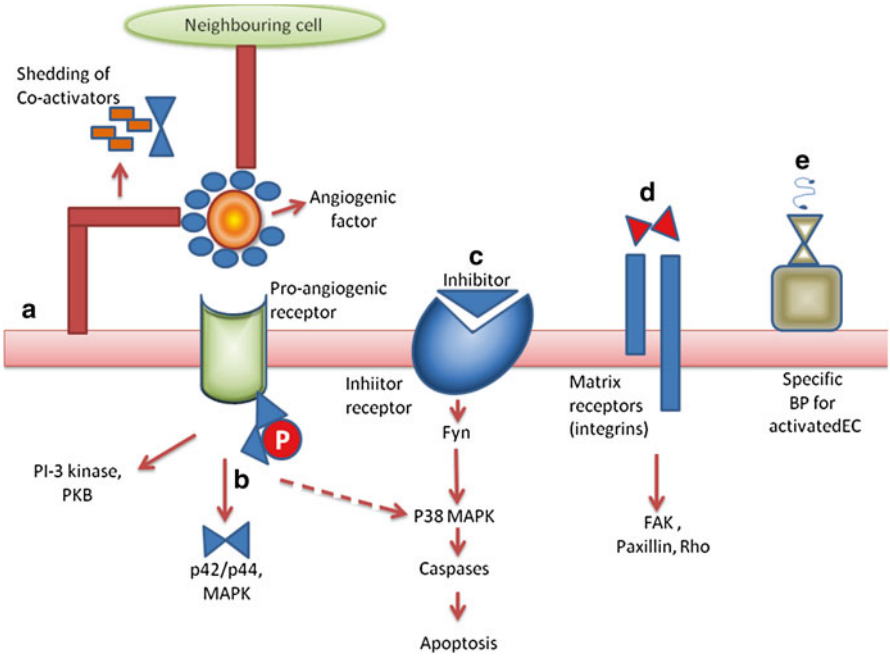

Fig. 2.6 Targeting tumor vasculature to inhibit angiogenesis. (**a**) Inhibition of binding to pro-angiogenic receptors and/or altering the interaction of angiogenic factors with co-receptors. (**b**) Penetration into the cells followed by binding with tyrosine kinase receptors (**c**) Direct activation of receptors, e.g., thrombospondin peptide mimetics (**d**) Extracellular matrix receptors, e.g., αvβ3 integrins. (**e**) Nonspecific inhibitors of proliferation

approved clinically and was followed by sunitinib, pazopanib, axitinib, etc. Interestingly, significantly higher therapeutic efficacy was noted upon combination of first approach with conventional chemotherapeutics which could be attributed to the *vascular normalization* capabilities of monoclonal antibodies leading to increased delivery of anticancer drugs to tumors. Bevacizumab when combined with irinotecan, fluorouracil, and leucovorin, improved therapeutic efficacy against metastatic colorectal cancer [110]. On similar line of action, combination of aflibercept with fluorouracil, leucovorin, and irinotecan drastically improves the survival in patients with metastatic colorectal cancer [111]. In contrast the actives in the second approach usually work best as single agent and the reasons for discrepancy is in part attributed to the tumor stromal architecture, intrinsic sensitivity, and resistance [112]; however, exact mechanisms are still under investigation [113].

Secondly, thrombospondin-1 represents the naturally occurring secretory angiogenic inhibitors and is principally responsible for organization of the perivascular matrix, endothelial cell adhesion and other process to counterfeit angiogenesis[114]. Numerous therapies have been employed for upregulation of thrombospondins such as metronomic dosing of antiangiogenic agents [115]. Cyclophosphamide has been found to upregulate circulating TSP-1 and not TSP-2 [116]. Notably, said approach

is also reported to sensitize the endothelial cells for TSP induced apoptosis mediated by Fas receptor overexpression [117]. In separate set of experiments, polymer implants containing TSP-2 overexpressed fibroblasts significantly increased therapeutic efficacy in the ovarian carcinoma and drastically higher levels of circulating TSP-2 were noted even after 5 weeks [118].

Thirdly, matrix metalloproteinases (MMPs) are the class of proteolytic enzymes primarily involved in the degradation of extracellular matrix and are part of well coordinated system of growth factors, inflammatory mediators and cell receptors [119]. These macromolecules are important for a variety of physiological functions including angiogenesis and hence, also explored as potential target in design antiangiogenic therapeutics [120]. Although dedicated MMPs inhibitors (MMPI) have not been successfully clinically, research is at advance stages to identify the specific properties which could be sought for rationalized development of MMPI leads.

Further, apart from VEGF based antiangiogenics which primarily block neovascularization, vascular disrupting agents represents a class of bioactives that selectively destroys the already formed tumor vessels by targeting dysmorphic endothelial cells [121]. Combretastatin A4 phosphate is a potent naturally occurring tubulin inhibitor and leads to vascular collapse and shut down of developed vessels and thus impart tumor regression [122]. Mechanistically, these are also reported to interfere with the functions of cadherins, thereby resulting in tumor necrosis. The other vascular disrupting agents include 5,6-dimethylxanthenone-4-acetic acid (DMXAA) that tends to increase nitric oxide, serotonin and tumor necrosis factor-α [123]; TZT-1027: dolastatin-10 analog; ZD6126: interferes with microtubules, Exherin®: cyclic pentapeptide; AVE8062A, ASA404, and MN-029.

2.5.3 Cancer Immunotherapy

The cancer immunotherapy seems to have a great potential in terms of clinical cancer therapy considering the remarkable progress in the field of molecular identifications of tumor antigens and increased understanding of various immunoregulatory pathways operative in the tumor microenvironment [124]. It is now well established that the tumor cells pose antigenicity and can be recognized by a variety of immune cells. Recently, the implementation of the shared tumor antigens has been largely replaced with the neoantigens that are generated by point mutations of genes specific to particular types of tumors. This has obvious advantage of improving the antitumor efficacy of the T cells by reducing the nonspecific interactions with that of normal host tissues and increasing the avidity of interactions among antigenic peptide and MHC molecule [125]. Exsome sequencing has been recently employed for defining the mutant antigens for a variety of cancers [126]. Furthermore, more than 20 antibodies are already approved for a series of disease conditions and a large number of them are under exhaustive investigation. Principally, these act through antibody dependent cellular cytotoxicity (ADCC) and complement-activation dependent cytotoxicity (CDC) for imparting cytotoxicity

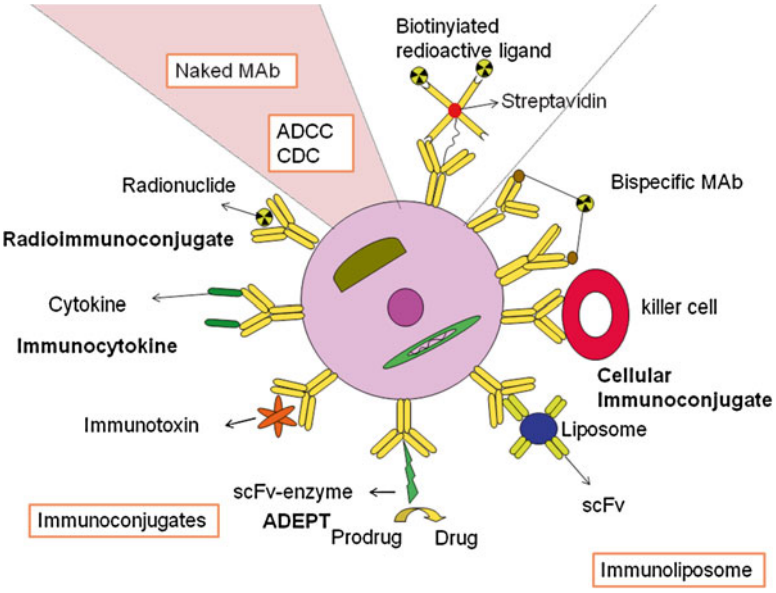

Fig. 2.7 Strategies for immunotherapy of cancer

and play a crucial role in efficient management of a variety of cancers [127]. In contrast to monotherapies, these antibodies are recently explored as adjuvants in drastically improving the therapeutic effectiveness of the conventional chemotherapies. Recently, antibody derived fragments have been explored to a greater extent as compared to that of parent whole antibodies considering the advantages such as manageable small size, relative ease in production, economic factors, and feasibility in antibody engineering for tailor-made applications. Figure 2.7 depicts various types of strategies that are currently employed for cancer immunotherapies [99, 128, 129].

2.5.3.1 Radioimmunotherapy (RAIT)

RAIT or Radioimmunoconjugates refer to the macromolecular entities where monoclonal antibodies (mAbs) are covalently attached to high linear energy transfer (LET) radionuclides. The radionuclides could either be alpha emitters such as ^{213}bismuth, ^{211}astatine, ^{223}radium, ^{149}terbium, ^{225}actinium, ^{227}thorium, ^{230}uranium or beta emitters such as ^{131}iodine, ^{90}yttrium, ^{60}cobalt, ^{99m}technetium capable of damaging DNA either by strand breakage or by other effects ultimately resulting in cell death. Concomitantly, the antibodies itself could also be detrimental to the cancerous cells by altering the signal transduction pathways such as alterations of signaling pathways and depression in gene expression by anti-CD20. Further, the conjugation of antibody with radiommunoconjugate leads to more *favorable biodistribution* [130]. Considering the huge potential of RAIT, a couple of products (Zevalin™,

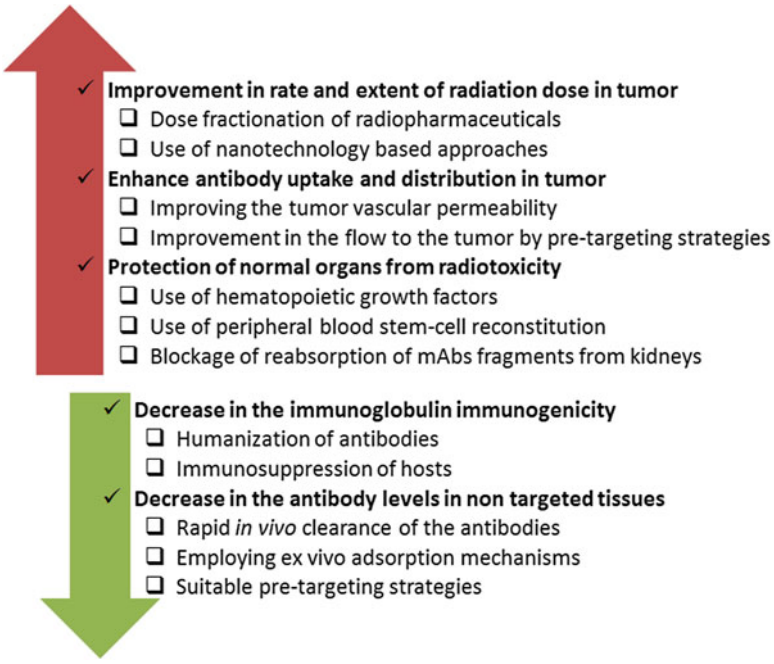

Fig. 2.8 Strategies to improve the therapeutic effectiveness of tumor radioimmunotherapy

Bexxar®, etc.) have already paved their way in clinical segment for treatment of non-Hodgkin lymphomas and many more are under clinical trials.

Since its genesis in early 1950s, the major applicability of RAIT has been hematological malignancies and few decades of research established preferential localization within tumor and subsequent therapeutic potential [131]. Drastic reduction in the bulky masses was noted in patients non-Hodgkin lymphomas treated with ^{131}I Lym-1 monoclonal antibodies [132]. Similar results were also observed with other antibodies such as anti-CD37 [133] and anti-CD20 [134]. The principal factors affecting RAIT includes type of mAbs, nature of radionuclide, and targeted host and tumor. The overall effectiveness of the RAIT further depends on the other factors such as rate and extent of dose administration, tissue penetration and sensitivity, location of target antigens (on tumor or within tumor), bone marrow toxicities, and tumor microenvironment [135]. Figure 2.8 depicts probable strategies that could be employed for improving the efficacy of RAIT whereas Table 2.4 reflects the exhaustive list of radionuclide and monoclonal antibodies employed for efficient management of cancer.

Recently, combination of neoadjuvant radiation therapy and chemotherapy are concomitantly employed for efficient management of cancers. Numerous clinical trials are presently undertaken in the direction of assessing the safety and antineoplastic potential of such RAIT, among which majority of trials encompasses combination of external-beam radiation therapy (EBRT), chemotherapeutics, and

Table 2.4 Exhaustive list of radionuclides and monoclonal antibodies employed in combination for efficient management of cancer

Radionuclide	Monoclonal antibody	Outcomes	Ref.
^{211}At	A33	Potential for treatment of micrometastases originating from colorectal carcinoma	[138]
^{213}Bi	Anti-EGFR	Improved therapeutic efficacy of radiation mediated by enhanced DNA damage	[139]
^{213}Bi	C595	Useful tool for the treatment of micro metastases or minimal residual disease (MRD)	[140]
^{213}Bi	Herceptin	Improved cell cytotoxicity against BT-474, SK-BR-3, and MDA-231 cell lines	[141]
^{213}Bi	MTAT	Inhibits lymph node micrometastases by induction of apoptosis	[142]
^{131}I	CC49 $(scFv)_2$	Provides a promising delivery vehicle for therapeutic applications	[143]
^{123}I	Tat-peptide	Significant G1–S phase arrest and efficient targeting of nuclear epitopes	[144]
^{131}I, ^{88}Y, ^{177}Lu, ^{186}Re	cG250	Improved stability and specific activity of the radionuclide conjugates	[145]
^{131}I, ^{88}Y, ^{188}Re	Mu-9 anti-CSAp	Promising results in the treatment of the GW-39 human colonic carcinoma	[146]
^{111}In	Anti-γH2AX	Significant increase in in vitro and in vivo anticancer efficacy	[147]
^{111}In	HuCC49ΔCH2/ cCC49	~4-fold appreciation in tumor to blood localization ratio of antibody conjugate	[148]
^{111}In	Mouse IgG (mIgG)	Tumor targeting was found to increase up to 15-fold	[149]
^{111}In	Trastuzumab	Shown potential HER2 specific targeting and radionuclide delivery ability	[150]
^{111}In	U36	Significantly higher uptake in tumor with a favorable biodistribution	[151]
^{111}In and ^{177}Lu	HER2/trastuzumab	Improved in vivo biodistribution profiles, tumor uptake and tumor-to-tissue activity	[152]
^{111}In and ^{90}Y	CC49	Significant reduction of extrahematopoietic toxicity	[153]
^{177}Lu	chCE7/ ChCE7agl	High and specific accumulation of radioactivity with enhanced antitumor efficacy	[154]

(continued)

Table 2.4 (continued)

Radionuclide	Monoclonal antibody	Outcomes	Ref.
^{177}Lu	M-BR96	Increased efficacy without significant increase in toxicity	[155]
^{212}Pb	Trastuzumab	Increased uptake rate in the tumor over a 72-h period with reduced systemic toxicity	[156]
^{149}Pm, ^{166}Ho, ^{177}Lu	CC49	Significant increase in the tumor uptake	[157]
^{99m}Tc	BIWA 1	Increased selectivity and tumor uptake with lower toxicity	[158]
^{227}Th	Rituximab	Novel approach for targeted delivery system	[159]
^{227}Th	Trastuzumab	Increased cumulative absorbed radiation dose to tumor by fractionation of the dosage	[160]
^{90}Y	HMFG1	Shown potential treatment efficacy at a dose of 18.5 mCi/m^2 with reduced toxicity	[161]

immunotherapy [136]. The principal component of the immunotherapy is usually a vascular endothelial growth factor (VEGF)-blocking antibody, whereas preferred chemotherapeutics is a platinum derivative such as cisplatin, oxaliplatin, etc. Table 2.5 lists the clinical trials presently undergoing on cancer RAIT [137].

With the advent of nanotechnology based approaches, the therapeutic effectiveness of the RAIT has been drastically improved. The said approach has been exclusively exploited in a variety of preclinical and clinical conditions and its potential has now been widely accepted. The principal amenities associated with these nanoparticles are their diverse functionalization potentials which help is improving the therapeutic efficacy and reducing the unwanted and toxic side effects to normal tissues. Figure 2.9 depicts the general architecture of these multifunctional nanoparticles and various advantages associated with said approach. In this context, a variety of nanocarriers have been fabricated till date for management of cancer and could broadly be categorized under the banner of either organic or inorganic nanocarriers [162]. Furthermore, employing the radiation chemistry, a radionuclide is conjugated to the antibody to form radionuclide–antibody conjugates which could be either conjugation of peptide ligands and antibodies or tumor targeted antibodies and radioisotopes [163]. The latter is then in incorporated into nanoparticles to yield targeted radioisotope labeled nanoparticles [164]. Table 2.6 list various nanocarrier based approaches employed for RAIT. The principal disadvantages with either plain radiation therapies or radio antibody conjugates are longer half lives, higher ϒ-emissions leading to undesirable side effects, inefficient internalization within lysosomes, mandate chelator requirements which often is associated with poor targeting or nonuniform dosimetry, etc. [165].

Table 2.5 Representative list of clinical trials comprising of radioimmunotherapy

Neoplasms	Phase	Radiation/antibody	Drug
Abdominal	I-III	^{90}Y-HMFG-1, ^{212}Pb-TCMC-Trastuzumab, ^{131}I-8H9	-
Adenocarcinoma	0-II	^{90}Y-m170, ^{90}Y-MN14, ^{18}F-FDG, cetuximab, filgrastim	Paclitaxel, docetaxel, cisplatin
Bronchial	0-II	^{131}I-L19SIP, ^{177}Lu-IMP-288, ^{111}In-IMP-288, antibody TF2, Pre-targeted radioimmuno-therapy, ^{90}Y-antiCEA cT84.66, ^{18}F-FDG, cetuximab	Cisplatin, docetaxel
Carcinoma	0-III	^{131}I-di-DTPA, carbon ion boost, ^{177}Lu-IMP-288, ^{111}In-IMP-288, ^{131}I-L19SIP, ^{90}Y-antiCEA cT84.66, ^{90}Y-DOTA anti-CEA M5A, ^{90}Y-HMFG-1, ^{111}In-MN14, antibody TF2, hMN14 (labetuzumab), cetuximab, bevacizumab, filgrastim	Doxorubicin hydrochloride, docetaxel, cisplatin, 5-fluorouracil, irinotecan hydrochloride, leucovorin calcium
CNS	I-II	^{131}I-3 F8, ^{131}I-8H9, ^{131}I-L19SIP	Cisplatin, lomustine, vincristine sulfate
Colorectal	I-II	^{177}Lu-IMP-288, ^{111}In-IMP-288, ^{90}Y-DOTA anti-CEA M5A, ^{90}Y-antiCEA cT84.66, ^{111}In-IMP-205xm734, ^{111}In-MN14, ^{90}Y-MN14, TF2, hMN14 (labetuzumab), filgrastim, bevacizumab	Oxaliplatin, leucovorin calcium, fluorouracil, gemcitabine, floxuridine, irinotecan, hydrochloride
Leukemia	I-II	^{90}Y-Epratuzumab, ^{111}In-LL2 IgG, ^{111}In-MN14, ^{131}I-Anti-B1, ^{131}I-BC8, ^{111}In-ibritumomab tiuxetan, ^{90}Y-ibritumomab tiuxetan, ^{131}I-tositumomab, rituximab, filgrastim, oprelvekin	Busulfan, cyclosporine, cyclophosphamide, etoposide, fludarabine phosphate, methotrexate, mycophenolate mofetil, melphalan, sirolimus, tacrolimus
Liver	I-II	^{90}Y-antiCEA cT84.66, ^{90}Y-DOTA anti-CEA M5A	Gemcitabine hydrochloride, floxuridine, fluorouracil, leucovorin calcium, oxaliplatin
Lymphomas	I-II	^{111}In-ibritumomab tiuxetan, ^{90}Y-ibritumomab tiuxetan, ^{131}I-tositumomab, ^{111}In-LL2 IgG, ^{90}Y-epratuzumab, ^{111}In-Lym-1, ^{90}Y-Lym-1, ^{111}In-MN14, rituximab, filgrastim	Cyclophosphamide, cisplatin, cytarabine, methylprednisolone, fludarabine phosphate, etoposide
Ovarian	I-III	^{212}Pb-TCMC-Trastuzumab, ^{90}Y-HMFG-1, ^{111}In-MN14, ^{90}Y-MN14, filgrastim	–
Prostrate	I	^{117}Lu-J591, ^{90}Y-m170, filgrastim	Cyclosporine, paclitaxel

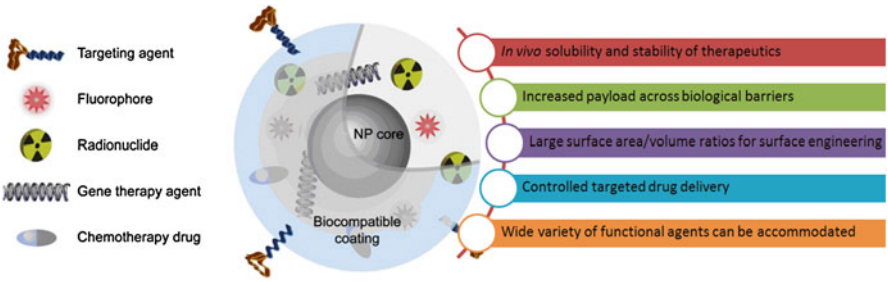

Fig. 2.9 General architecture of multifunctional nanoparticles and associated principal advantages. Adapted and modified with permission from [166]

2.5.3.2 Immunotoxins

Immunotoxins represents a group of macromolecular species essentially comprising toxin conjugates and targeting antibodies linked via gene fusions, peptide bonds, disulfide bonds, thioether bonds, etc. [180]. Upon successful internalization within target cells, the immunotoxins releases toxin by a variety of mechanisms such as degradation by proteases, reduction of disulfide bonds, or acid hydrolysis (Fig. 2.10). Primarily bacterial toxins such as diphtheria toxins, *Pseudomonas* exotoxin, and plant toxins such as ricin, modeccin, abrin, etc. are employed as toxin conjugates. These are usually proteinaceous in nature and are considered as highly potent, owing to which even limited availability at site of action is sufficient for execution of cellular responses [181]. Once inside the cells, these tend to inhibit the protein synthesis pathways and result in cell cytotoxicity. Although fascinating, the clinical intervention of immunotoxins is a great challenge owing to associated limitations such as poor antigen specificity, lower cytotoxicity potential, nonspecific side effects, immunogenicity, and manufacturing complications. Recently, in the last couple of decades, strategic developments in the field of immunotoxins have been noticed. Started with its genesis from isolation of potential toxins in early 1970s, a variety of immunotoxins have made their way to clinical trials successfully clearing in vitro tissue culture experiments and in vivo preclinical testing. One product, DT-IL2 (denileukin diftitox, Ontak™), has been clinically approved for human use and specifically targets IL-2 receptors [182].

The targeting antibodies could either be whole antibody or small fragments. In the latter case, A chain subunit of the toxin is linked to the monoclonal antibody and are referred to as A chain immunotoxins. A principal advantage associated with such A chain immunotoxins is lower nonspecific side effects in in vivo setting; however, in contrast some significant compromise in overall targeting potential and cell cytotoxicity was noted as compared to whole antibody immunotoxins [183]. The results are indicative of the crucial role of B chain in improving the interactions of immunotoxins with target cells and preferential assistance in entry to cytosol. Furthermore, considering the pharmacokinetic perspectives, the larger the constructs, the longer the circulation half-life. Furthermore, classical to any anticancer

Table 2.6 List of nanocarrier based approaches employed for RAIT

Radionuclide	Nanocarrier approach	Functionalization	Outcomes	Ref.
^{90}Y	Polymerized liposomes	Integrin antagonist; anti-Flk-1 mAb	Significant tumor growth delay in K1735-M2 and CT-26 tumors	[167]
^{99m}Tc	PEGylated liposomes	MIBI	Twofold higher uptake in MCF-7 ras tumor bearing mice	[168]
^{111}In, ^{88}Y	PAMAM dendrimer	Humanized anti-TacIgG (HuTac)	Significant differences in the biodistribution patterns of the saturated and unsaturated dendrimers were noted	[169]
^{111}In	PEGylated PE micelles	2C5	Significantly higher tumor accumulation in murine LLC	[170]
^{111}In	Albumin nanoparticles	RGDGSSV peptide, fibrinogen	Significant retardation in tumor growth and tumor specific reduction blood flow to B16F0 hind limb tumors	[171]
^{111}In	Perfluorocarbon nanoparticles	$\alpha\nu\beta_3$-integrin binding[a]	Fourfold higher mean tumor activity in Vx-2 tumor bearing rabbits as compared to nontargeted controls	[172]
^{188}Re	Liposomes	Doxorubicin	Significant increase in the therapeutic efficacy against C26 murine solid tumor animal model	[173]
^{111}In	Nanocapsules	Polysaccharides	Significant retardation in the clearance rate and preferential biodistribution within lymphatic system	[174]
^{131}I	Dextran magnetic nanoparticles	Sc-7269	Significant tumor growth delay and tumor inhibition rate were noted without any compromise in safety profile	[175]
^{90}Y	Apoferritin	Biotin	Significant pre-targeting capabilities	[176]
^{125m}Te	ZnS nanoparticles	mAb 201B	Significantly higher localization within lungs	[177]
^{64}Cu	Carbon nanotubes	RGD	Significantly higher tumor uptake with minimal renal clearance	[178]
^{225}Ac	PEGylated liposomes	PSMA J591 antibody, A10 PSMA aptamer	Significantly higher cytotoxicity against PSMA overexpressing human LNCaP cells, rat Mat-Lu cells and HUVEC cells	[179]

[a]Perfluorocarbon nanoparticles bind to $\alpha_v\beta_3$-integrin receptors

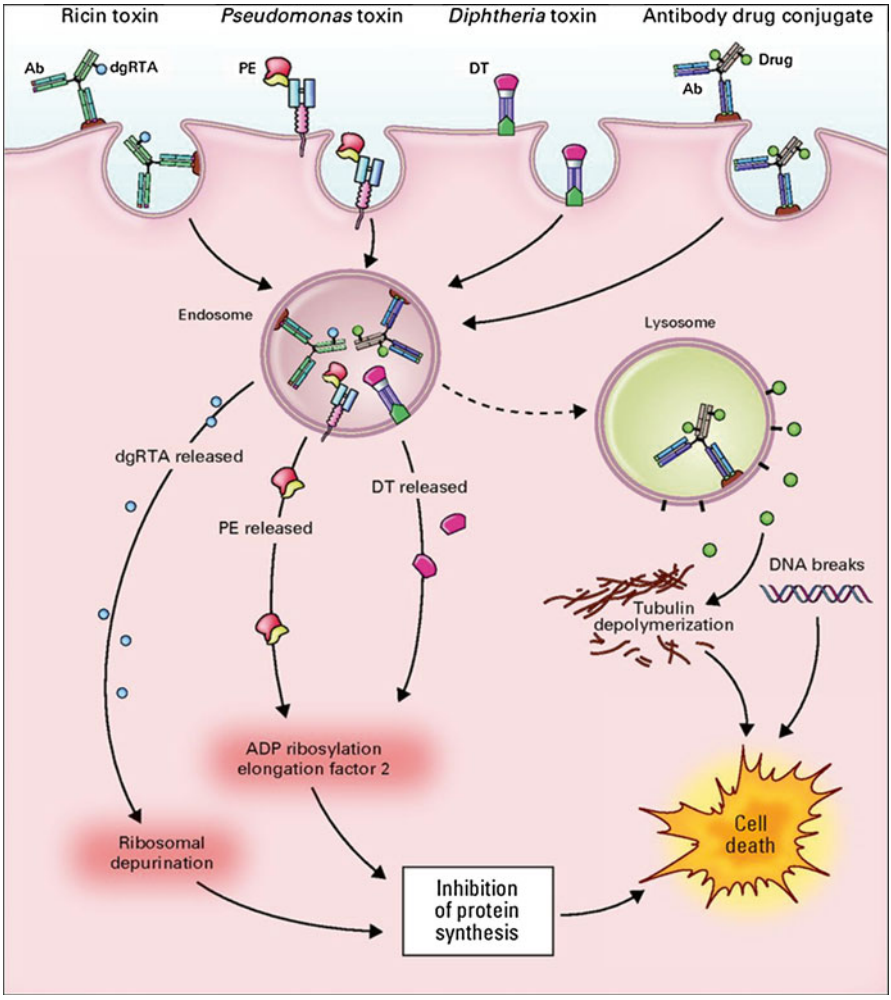

Fig. 2.10 Mechanisms of action of monoclonal antibody (Ab) conjugates. Monoclonal antibodies and their fragments can be conjugated or linked to cytotoxic agents. Chemotherapy and toxin conjugates must be internalized via receptor-mediated endocytosis, whereas internalization is not required for radioisotope conjugates. After internalization, the active cytotoxic component is released and mediates cell death. Ricin-based immunotoxins depurinate ribosomal RNA and inhibit protein synthesis. Pseudomonas (PE)- and diphtheria (DT)-derived immunotoxins ADP ribosylate elongation factor-2 and inhibit protein synthesis. Antibody drug conjugates mediate cytotoxicity by drug-specific actions (e.g., targeting tubulin by maytansin and auristatin, and induction of DNA breaks by calicheamicin). *dgRTA* deglycosylated ricin A chain. Reproduced from ref. [186]

chemotherapy, the potency of immunotoxins could be drastically improved by coadministration with *enhancing agents*. Such improvements in the entry of immunotoxins within the cells could be facilitated by understanding the vesicular entry systems classical to proteins, employing the pathways adapted by natural toxins

during pathological conditions, and exploiting the structure–function relationships of natural toxins in designing the entry systems [184]. Classically, pharmacologically active molecules such as lysosomotropic amines or carboxylic ionophores have also been employed as enhancing agents [185].

Concomitant with high therapeutic potency, there also lie numerous side effects associated with immunotoxins. These includes flu-like syndrome, vascular leak syndrome, infusion related hypersensitive reactions, and transient increase in the levels of hepatic toxicity markers such as transaminases owing to preferential processing of immunotoxins in liver [33]. Furthermore, immunotoxicity can also occur either due to monoclonal antibodies or toxins which could be marginally circumvented by employing antibody alterations or humanization.

Table 2.7 list various immunotoxins employed for management of cancer. Particularly, deglycosylated ricin A (dgA) chains have been particularly explored in a wide array of malignancies considering diminished hepatotoxicity (owing overexpression of mannose receptors in liver) classically observed with anti-B4 blocked ricin [187]. The lateris also associated with significant human anti-mouse antibody (HAMA), anti-ricin (HARA) immune responses and vascular leak syndrome [188]. In purview of this, the particular amino acid sequences in toxins responsible for both therapeutic effects and detrimental side effects have been identified and employed [189, 190]. The reduction in HARA immune responses could be mediated by employing the PEGylated ricin as compared to plain ricin [191]. Interestingly, PEGylation does not affect the inhibition of protein synthesis pathways by ricin.

Notably, immunotoxins usually lacks any bystander effects. However, considering the very therapeutic potency, this property could be fruitfully exploited in combination therapeutic regimen to combat minimal residual diseases, particularly hematological malignancies. Immunotoxins along with RAIT have been successfully employed for treating disseminated human B-cell lymphoma in immunodeficient mice model and curative therapeutic regimen was observed by optimizing the temporal order of administration without any life threatening vascular leak syndrome (Fig. 2.11) [209].

2.5.3.3 Immunocytokines

Immunocytokines represents yet another important type of macromolecular species employed for efficient management of cancer. These are fusion proteins essentially comprise monoclonal antibody and cytokine. Cytokines are referred to as cell signaling molecules responsible for cell–cell communication and mediate a variety of humoral and cellular immune responses to maintain homeostasis [210]. These include interleukins, interferons, chemokines, colony stimulating factors, lymphokines, and tumor necrosis factors, to name a few. Mechanistically, immunocytokines tend to accelerate the compromised tumor immune responses and hence the immunocytokines therapy seems to be most promising as compared to any other immunotherapies (Fig. 2.12). Considering such potential, IL-2 based immunocytokines therapy has been clinically approved for treatment of advanced stages of

Table 2.7 Immunotoxins employed for efficient management of cancer

Toxin conjugate	Monoclonal antibody	Remarks	Ref.
Diphtheria toxin (DT)	Trastuzumab (Herceptin)	Trastuzumab-DT conjugates exhibited significant killing of SK-BR-3 cells	[192]
Diphtheria toxin mutant (CRM9)	Anti-vascular endothelial growth factor (VEGF)	Markedly higher cell cytotoxicity than control groups	[193]
Gelonin	Monoclonal antibody 31 (MOC31)	MOC31-gelonin and 5-aminolevulinic acid (5-ALA) combination induced synergistic cytotoxic effect against the WiDr cells via enhanced photo chemical internalization as a result of protoporphyrin IX (PpIX)	[194]
Granzyme M	Humanized single-chain antibody fragment (scFv) H22	Specific and efficient toxicity upon binding to CD64, an FcγRI receptor overexpressed on activated myeloid cells and leukemic cells	[195]
Listeriolysin O (LLO)	B3	Specific elimination of antigen positive MCF7 cells with up to 80–250-fold less sensitivity towards antigen negative cell lines	[196]
LysPE38QQR (truncated form of Pseudomonas exotoxin)	K1 (murine IgG1)	Exhibits higher toxicity against mesothelin positive A431-K5 cells	[197]
Mutant Pseudomonas exotoxin A (ETA')	anti-EGFR 425(scFv)	Higher binding activity and specificity of towards EGFR-positive pancreatic carcinoma cell line L3.6p1	[198]
Mutant Pseudomonas exotoxin 38 (PE38)	B3	About 12-fold higher cytotoxicity on CRL1739 cell lines	[199]
Pseudomonas exotoxin (PE38)	Mutant MR1(Fv)	Increased affinity and cytotoxic activity	[200]
Pseudomonas exotoxin 38 (PE38)	RFB4	Exhibited fivefold to tenfold increase in activity on various CD22-positive cell lines and up to 50 times more cytotoxic to cells from patients with chronic lymphocytic leukemia and hairy-cell leukemia	[201]
Pseudomonas exotoxin 40, (PE40)	Humanized anti-CEA antibody (hMN14)	hMN14(Fv)-PE40 showed specific growth suppression of CEA expressing cell lines MIP-CEA (high CEA) and LS174T (moderate CEA) with IC_{50s} of 12 ng/mL (0.2 nM) and 69 ng/mL (1.1 nM) respectively with reduced toxicity towards normal tissues	[202]
Pseudomonas exotoxin A	anti-CD22	Remarkable increase in thermal stability and an enhanced resistance to trypsin degradation	[203]

(continued)

Table 2.7 (continued)

Toxin conjugate	Monoclonal antibody	Remarks	Ref.
Pseudomonas exotoxin A ETA	HER2-specific single-chain antibody scFv(FRP5)	scFv(FRP5)-ETA showed specifically higher cytotoxicity towards HER2 positive cell lines LNCaP	[204]
rAbrin	mAb F1G4	Immunotoxin mAb F1G4-rABRa-A, inhibits protein synthesis specifically on cells expressing the gonadotropin releasing hormone receptor and also it exhibited differences in the kinetics of inhibition of protein synthesis, in comparison to abrin, which was attributed to differences in internalization and trafficking of F1G4-rABRa-A within the conjugate	[205]
Recombinant gelonin toxin (rGel)	FGFR3-specific Fv fragments (3C)	3C/rGel fusion showed an significant reduction of IC50 value up to 200 nmol/L against cells compared with 1, 500 nmol/L for free rGel	[206]
Ricin A	(anti-PSMA) monoclonals (J591, PEQ226.5, and PM2P079.1	Various immunotoxins showed an significant reduction PSMA + cells with IC50 value in nanomolar range (IC_{50s} of 1.6–99 ng/mL) and complete eradication with J591-smpt-nRTA with IC_{50} of 0.35–31.7 ng/mL	[207]
Saporin	Trastuzumab (Herceptin) and cetuximab (Erbitux)	Trastuzumab (Herceptin) and cetuximab (Erbitux) were conjugated via cleavable disulfide bonds to the plant derived toxin saporin shown to have overcome the present limitations of therapeutic antibodies with a higher antitumoral efficacy via endosomal/lysosomal release of the toxin moiety	[208]

melanoma and renal carcinoma [211]. In addition, some allied pharmacological activities of cytokines have also been noted such as inhibition of tumor vasculature by tumor necrosis factors [212]. Considering the physiological functions of cytokines as either auto or paracrine factors, pretty high concentrations needs to achieved in the close proximities of site of action, i.e., producing cells. However, the clinical limitations does not permit such high dose administrations systemically [213] and hence the classical approaches includes either direct injection within solid tumor [214] or localized treatment such as isolated limb perfusion of tumor necrosis factor [215]. Unfortunately, the said approaches does not comply in most of the malignancies at the advanced stages and hence the concept of immunocytokines emerged wherein a tumor antibody is attached to the cytokine to achieve preferential higher tumor concentrations and hence drastically reduce toxic side effects to normal tissues. Furthermore, drastic improvements in the pharmacokinetics of cytokines could be achieved by said strategy [210].

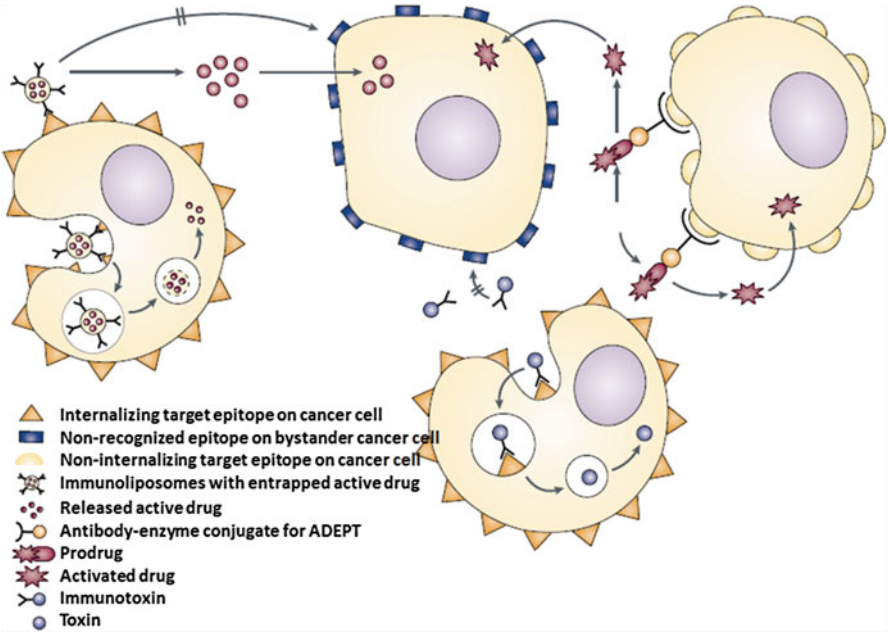

Fig. 2.11 Internalization of ligand targeted therapeutics and the "bystander effect." (**a**) Binding of the ligand-targeted therapeutics (LTTs) to their target epitopes will, in the case of some antibodies, promote receptor-mediated internalization of the LTT and, following release of the therapeutic intracellularly, lead to cytotoxicity (e.g., immunoliposomes and immunotoxins). (**b**) Binding of LTTs linked to noninternalizing antibodies will result in the LTT remaining attached at the target-cell surface (e.g., ADEPT (antibody-directed enzyme–prodrug therapy)). (**c**) All the cancer cells will preferably express the target epitope; however, some of the cancer cells might not. Drug that is released into the tumour interstitial space might be taken up non-selectively by cancer cells that do not express the target epitope; this results in cytotoxicity by the "bystander effect" (e.g., immunoliposomes and ADEPT). (**d**) Immunotoxins must be internalized to show cytotoxicity, so no opportunity for a bystander effects exists. Reproduced with permission from ref. [33]

Interleukins, especially IL-2, have been exhaustively studied for tumor immunotherapy. High dose IL-2 was the pioneer immunotherapy approved for treatment of melanomas and had complete response of ~6 % and partial response of ~10 % [216]. However, it is associated with severe side effects such as vascular leak syndrome, hypersensitivity reactions, etc. In contrast, the immunocytokines therapy demonstrated remarkably higher tumor accumulation of cytokines without notable compromise in safety profile [217]. In another set of experiments, 20-fold higher potency was noted for antibody targeted IL-12 in contrast to that of naked IL-12 [218]. On these grounds, a variety of immunocytokines based products such as Proleukin™, Aldesleukin™ comprising IL-2; Beromun™ comprising TNF-α; Roferon-A™ and Intron-A™ comprising interferon-α2; and Leukine™ and Leucomax™ comprising granulocyte-macrophage colony-stimulating factor (GMCSF) have been approved clinically [219]. In parallel, the exploration of

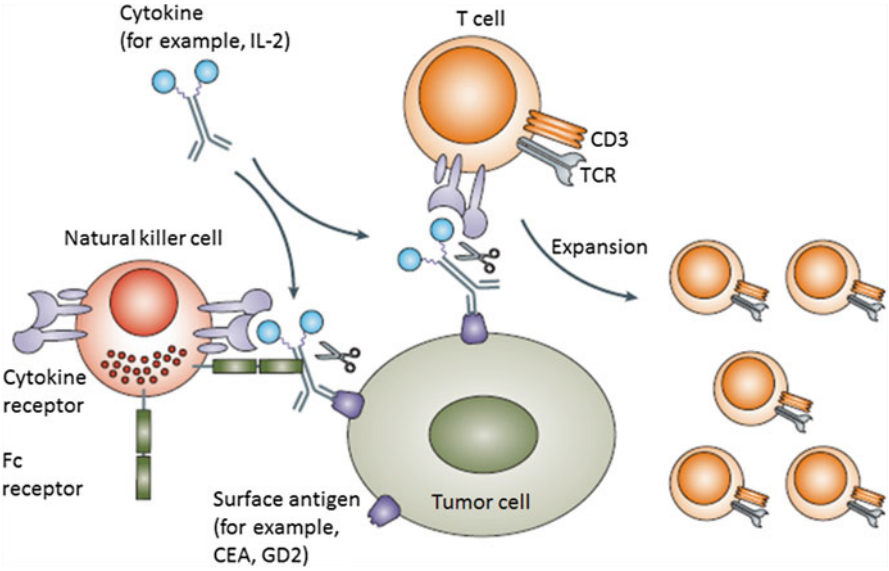

Fig. 2.12 Working mechanism of immunocytokines exemplified for tumor targeted IL-2. A monoclonal antibody specific for a tumor-associated antigen allows the enrichment of cytokines in the tumor microenvironment. In the case of interleukin-2 (IL-2) it enhances antibody-dependent cellular cytotoxicity mediated by Fc-receptor positive effector cells such as natural killer cells. In addition, tumor-targeted IL-2 stimulates T cells to expand and attack the tumor. High concentrations of plasmin at the tumor site enable the cleavage of IL-2 from the fusion protein through the plasmin cutting site within the linker (depicted in the figure by scissors). Reproduced from ref. [211]

cytokines as vaccine adjuvants is also in move. Upon combination with dendritic cells interesting memory immune responses were noted in the sense that site specific memory response mediated by effector memory T cells was executed whereas the central memory T cells were not formed [220]. The hypothesis was based on the fact that in rechallenge test the no tumor relapse was observed in organ in concern whereas tumor was induced in other organs. Recently, complete eradication in the B-cell lymphoma xenograft was observed upon treatment with combination of rituximab and L19-IL2 [221]. Similar results were also noted when L19-IL2 combined with (Cytotoxic T-Lymphocyte antigen 4) CTLA-4 blockade or L19-TNF [222]. Of note, not all cytokines exhibit antitumor effects, lymphotoxins represent a class of cytokines leading to physiology of tumorigenesis mediated by inflammatory pathways [223]. The said discrepancy could possibly be attributed to the typical structural motif of lymphotoxins which is trimeric in contrast to that of heterodimeric structure of potent antitumor cytokines such as IL-12 [185]. Hence, while employing immunocytokines based cancer therapy, the alterations in allied pathways and its clinical implications should be considered. Table 2.8 depicts various immunocytokines employed for cancer immunotherapy.

Table 2.8 List of immunocytokines employed for cancer immunotherapy

Cytokine	Monoclonal antibody	Outcomes	Ref.
IL-2	2aG4, PS targeting antibody	80 % of mice inoculated with 2aG4-IL2/4 T1 vaccine survived free of tumor and significantly increased 4 T1 specific cytotoxicity and ability to secrete interferon gamma (IFNγ)	[224]
IL-2	14G2A antibody	Uterine leiomyosarcoma diffusely expressed GD2 and binds the therapeutic immunocytokine14.18-IL2 and shown to be a potential target for effective management of aggressive tumors	[225]
IL-2	F8 antibody	F8-IL2 effectively inhibited the growth of EDA-Fn-expressing melanomas in combination with paclitaxel as a result of recruitment of F8-IL2-induced natural killer (NK) cells to the tumor via paclitaxel mediated enhanced tumor perfusion and permeability.	[226]
IL-2	F16 antibody	Selective tumor staining of F16 and preferential tumor accumulation of radiolabeled F16-IL2	[227]
IL-2	L19 and anti-CTLA-4 antibody	L19-IL2 exhibited complete tumor eradications when used in combination with CTLA-4 blockade	[222]
IL-2	F8 antibody	28 % cure rate and substantial tumor growth retardation were observed for the combination of sunitinib with F8-IL2 immunocytokine	[228]
IL-2	hu14.18 antibody	Patients treated with hu14.18-IL2 immunocytokine developed anti-idiotypic antibodies and anti-Fc-IL2 antibodies	[229]
IL2	huKS antibody	Paclitaxel and cyclophosphamide followed by huKS-IL2 resulted in enhanced antitumor responses against CT26/KSA colon, 4 T1/KSA mammary and LLCKSA Lewis lung carcinomas due to increased uptake of the huKS-IL2 immunocytokine into the tumor microenvironment by the virtue of reduced diffusion barrier by drug therapy	[230]
IL2	huKS antibody	Significantly increased (complete tumor resolution in 50 % of mice) the antitumor effect of RFA (Radiofrequency ablation) by combining it with huKS-IL2. Immunocytokine also showed antitumor effects against distant untreated tumor and greater proportion of cytokine-producing CD4 T cells and CD8 T cells	[231]
IL-7	F8 antibody	Improved tumor targeting performance with tumor: blood ratio = 16:1	[232]
IL-12	NHS76 antibody	Significant increase in the toxicity profile of NHS-IL12 was achieved as a result of attenuated IFN gamma production, selective targeting and longer half-life	[233]

(continued)

Table 2.8 (continued)

Cytokine	Monoclonal antibody	Outcomes	Ref.
IL-12	SS1 Fv	IL12-SS1 (Fv) immunocytokine significantly inhibited human tumor expressing mesothelin proteins, i.e., malignant mesothelioma (NCI-H226) and ovarian (OVCAR-3) cells as well as recombinant mesothelin on A431/H9 cells	[234]
IL-12	Recombinant human antibody fragment L19	Antitumor activity of EMD 521873 (Selectikine immunocytokine) was reported in heterogeneous patient population as prolonged disease stabilisation and a transient drop in tumor markers and also it was found that at all dose-levels there were transient increase in total lymphocyte, eosinophil, and monocyte counts	[235]
IL-15, GMCSF	L19 antibody	L19-IL-15 and L19-GM-CSF displayed a potent antitumor activity via CD8+ T cells	[236]
IFN-α	hRS7, hMN 15, hL243, and c225 antibodies	Up to 1,000-fold improved anti-proliferative potency of IFN-λ1, when tethered with antibodies hRS7, hMN 15, hL243, and c225, was demonstrated against targeted cancer cell lines along with increased antiviral activity against encephalomyocarditis virus and hepatitis C virus IFN-λ1 via (15)-λ1 or (c225)-λ1 respectively	[237]
IFN-γ	Anti-CD70 antibody	Anti-CD70 IFN-γ immunocytokines displayed high levels of species specific IFN-γ activity and selective binding to CD70 on human RCC cells and higher tumoricidal activity by the virtue of RIP1-dependent necrosis in RCC cells in the presence of bortezomib	[238]
TNF	scFv23	scFv23/TNF was found to be highly cytotoxic to TNF-resistant HER-2/neu-expressing pancreatic cancer cell lines and demonstrated a synergistic cytotoxic effect with 5-fluorouracil (5-FU) by the virtue of downregulation of HER-2/neu, p-Akt, Bcl-2 and upregulation of TNF-R1, caspase-8, and caspase-3	[239]

2.5.3.4 Antibody Directed Enzyme Prodrug Therapy (ADEPT)

ADEPT represents an improved version of cancer immunotherapy among the above discussed strategies for efficient management of advanced malignancies and solid tumors. Classical immunotherapies face challenges such as limited penetrating capabilities, need for internalization, antigen heterogeneity, nonuniform drug release and subsequent altered potency levels. Principally, ADEPT operates via usage of antibodies specific for tumor agents that are covalently linked to enzyme in the concern. In the first step, such antibody–enzyme conjugate is administered to the patients and allowed to preferentially localize in the vicinities of tumor. Once the unbound conjugates are cleared of the body by a variety of means, a prodrug

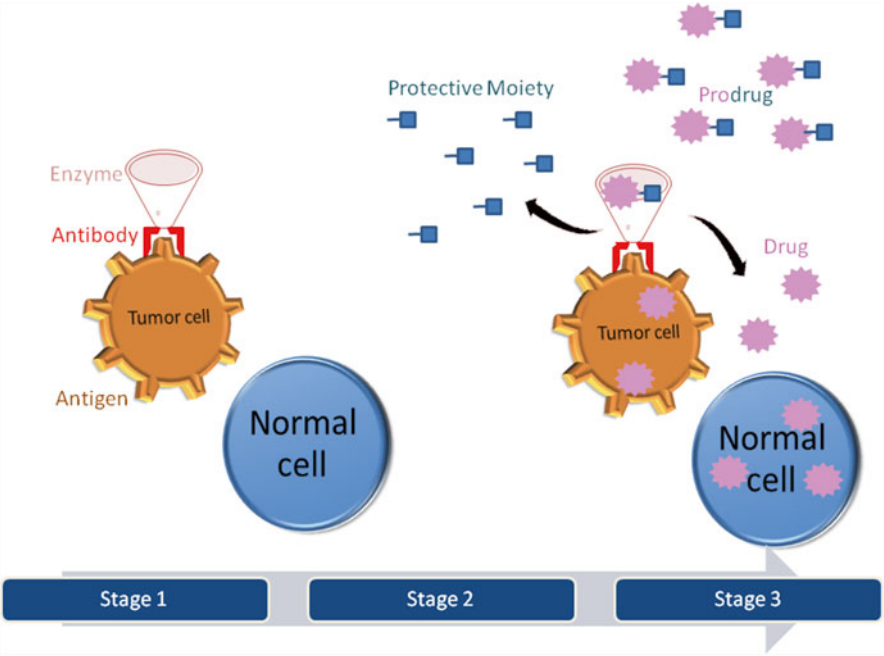

Fig. 2.13 Basic principle of ADEPT. *Stage 1*, administered AEC is allowed to localize at site of action. *Stage 2* the excess circulating prodrug is cleared of the blood. *Stage 3* nontoxic prodrug is administered that specifically generates active at site of action

sensitive to pre-targeted enzyme is administered which will generate very high local concentrations of active drug in the extracellular regions of tumor. The active drug can then be passively internalized within cells and impart cytotoxicity (Fig. 2.13) [240]. One of the promising advantages is bystander effects which also impart cytotoxicity to antigen negative cells (Fig. 2.11) [241].

The principal components of ADEPT include target, antibody, enzyme, abenzymes/catalytic antibodies, and prodrugs [242]. Firstly, target should usually be native to the tumor and should have minimal expression over normal tissues. However, it is practically difficult to find such targets and hence care should be taken that normal expression of target is at minimal at least on the vital organs so to avoid any irreparable damages to the critical body organs. However, heterogeneity among in antigen expression in epithelial tumors is quite common. Furthermore, the internalization of the antibody–enzyme conjugate should be avoided so as to avoid failure of therapy. The additional advantage of the surface linked enzyme is rapid conversion of many prodrug molecules and higher tumoral concentrations. While selecting target antigen, care should also be taken that it should not be secretory in nature; however, there have been some exceptions such as ADEPT in the case of human chorionic gonadotrophin (hCG) producing tumors was successful in spite of higher concentrations of hCG in central compartment [243].

Secondly, antibody plays an important role in preferentially localizing the enzymes and hence the therapeutics at the target location so as to achieve the maximum benefits, per se. This retention of enzymes at the target site may extend up to several days. In this regard, considering the binding affinity of the employed antibody, interplay of antibody–enzyme conjugate in the vicinity of tumor and in central compartment could be modulated with ideal situation being nil antibody–enzyme conjugate in central compartment for achieving maximum therapeutic efficacy. Such highly stable antibody complex could be achieved by employing class I or II IgG monoclonal antibodies. Furthermore, this balance could be modulated in a better way by usage of antibody fragments such as F(ab)′2 which are associated with principal advantages of rapid clearance from the central compartment imparting better targeting potential to the tumors; however, the binding affinity, size of fragment, renal clearance and hence total available enzymatic concentrations at the site of action, etc. should be taken into account while comparing it with whole antibodies [244].

Thirdly, enzyme has principal role for success of ADEPT. However, there lies certain restriction on type of enzymes which could be used such as human enzymes exhibit wide normal tissue distribution while nonhuman enzymes face problems of immunogenicity and possible human isoforms in physiological conditions. Among the available options, bacterial enzymes are explored considering their potential efficiency to a greater extent; however, their immunogenicity is debatable. In purview of decreasing the immunogenicity, efforts are being to identify the responsible amino acid sequences and their suitable replacement in modified enzymes [245, 246]. In this regard, yet another interesting concept of preparing mutant human enzymes is prevailing which will enable the prodrug processing only by mutated enzymes, whereas original human forms of these enzymes remain inactive, e.g., T268G mutant of human carboxypeptidase A1 for prodrugs of methotrexate [247]. Table 2.9 lists various enzymes employed for ADEPT.

Fourthly, the concept of catalytic antibodies is also emerging as promising component of ADEPT with the principal advantage of overcoming immunogenicity related problems [267]. Conceptually these are improved version of therapeutic antibodies which are equipped with additional enzymatic catalytic power (Fig. 2.14). The most classical methods to prepare such catalytic antibodies are transition state analogue approach, hapten substrate approach and reactive immunization approach. Their immense clinical relevance arises from the promising advantages such as improved circulation half-life in central compartment, increased specificity and affinity for the target and prodrugs, and feasibility to exploit antibody/genetic engineering along with the classical advantage of low immunogenicity. Some examples of catalytic antibodies include 38C2 for aldolase activity; 84G3, 85H6, 90G8, and VHHC10 for aliinase activity; 3D8-VL for mRNA of HER2 hydrolysis; A17 for degradation of organophosphate compounds, ETNF-6-H for hydrolysis of TNF-α, etc. Recently, cell targeting and prodrug activation capabilities of the next generation therapeutic antibody, catalytic Ab 38C2, have been established in the case of selective tumor immunotherapy with doxorubicin prodrugs [268].

Table 2.9 List of enzymes employed for ADEPT based cancer immunotherapy

Enzyme	Characteristics	Ref.
β-glucosidase	β-glucosidase conjugated with tumor associated monoclonal antibody HMFG1 enhances the cytotoxic effect of amygdalin by 36-fold while increased rate of survival of U87MG (HMFG1-negative) cells	[248]
β-glucuronidase	After fusion with a humanized single-chain antibody (scFv) of mAb CC49 to S2 (a human β-glucuronidase (hβG) variant) displays enhanced human enzyme catalytic activity and more effective in killing antigen-positive cancer cells	[249]
β-Lactamase	Fusion protein TAB2.5 (conjugation of CC49 and β-lactamase) showed prolonged retention (T1/2 = 36.9 h) with tumor to plasma ratios of up to 1,000	[250]
β-Lactamases	When fused with ACDCRGDCFCG peptide (RGD4C), it found to be active for specificity in MCF-7 cell lines	[251]
Carboxypeptidase-A	Anti-seminoprotein (SM) single-chain antibody/human carboxy peptidase A fusion protein (scFv/hCPA) mediated hydrolysis of methotrexate prodrug increased cytotoxicity up to 1,000-fold with no systemic toxicity of the prodrug	[252]
Carboxypeptidase A	HuA33 antibody–carboxy peptidase A (acts on methotrexate phenylalanine prodrug) demonstrates higher specificity of tumor uptake with eightfold reduced cytotoxicity (LD50 of MTX) to non-tumor sites	[253]
Carboxypeptidase G2 (CPG2)	Conjugation of CPG2 with SB43 anti-carcinoembryonic antigen antibody fragment A5B7-F(ab′)$_2$ shown to reduce the percentage of injected dose per gram in blood and a tumor-to-blood ratio of 45: 1 at 24 h, which increased to 100: 1 at 72 h	[254]
Carboxypeptidase G2 (CPG2)	F(ab′) [SUB2] anti-CEA antibody A5B7 and the bacterial enzyme CPG2 conjugates converts ZD2767P (4-[N,N-bis(2-iodoethyl) amino] phenoxycarbonyl L-glutamic acid) to ZD2767 (bifunctional alkylating agent) at the tumor site which resulted in 229-fold increase in activity.	[255]
Carboxypeptidase G2 (CPG2)	Anti-carcinoembryonic A5B7 conjugated to the bacterial enzyme carboxypeptidase G2 acts on the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid resulted in significantly enhanced tumor growth with no concomitant increase in systemic toxicity	[256]
Carboxypeptidase G2 (CPG2)	MFECP, a recombinant fusion protein of CP with MFE-23, a single chain Fv (scFv) antibody, modified to hexahistidine tag (His-tag) MFECP found to have significantly reduced human antibody response	[257]
Cytosine deaminase (CD)	Fused LinkCD, hyaluronan binding domain of TSG-6 (Link) and yeast cytosine deaminase (CD) with an N-terminal His(×6) tag shown to have increased duration of the enzyme activity and significant tumor size reduction in animals that received LinkCD/5-FC treatment	[258]

(continued)

Table 2.9 (continued)

Enzyme	Characteristics	Ref.
Cytosine deaminase (CD)	Fusion of anti-gpA33 single chain fragment, A33scFv, with cytosine deaminase from yeast (CDy), which converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), results demonstrate bifunctional activity of A33scFv::CDy fusion protein which is equally cytotoxic to equimolar amounts of 5-FU	[259]
Human β-glucuronidase (βG)	chTNT-3 and β-glucuronidase enzyme fusion protein (acts on doxorubicin glucuronide prodrug), specifically localize to tumor sites with rapid clearance from blood and normal tissues	[260]
Human β-glucuronidase (βG)	It was found that ortho-substituted phenyl carbamates (i.e., N-[4-O-(Methyl-βD-glucopyranosyluronate)-3 nitrobenzyloxycarbonyl] doxorubicin) are better substrates for fusion protein human β-glucuronidase-humanized CEA than the corresponding para-substituted analogues	[261]
Human β-glucuronidase (βG)	Anti-CD20 mouse monoclonal antibody (MoAb) 1H4 and human β-glucuronidase activates nontoxic prodrug *N*-[4-doxorubicin-*N*-carbonyl(-oxymethyl) phenyl] O-β-glucuronylcarbamate to doxorubicin at the tumor site	[262]
Mutant human purine nucleoside phosphorylase	Double mutant with amino acid substitutions E201Q:N243D (hDM) is the most efficient in cleaving (deoxy) adenosine-based prodrugs, i.e., 2-fluoro-2′-deoxyadenosine to a cytotoxic drug	[263]
Mutant human carboxypeptidase A1, changed at position 268 from the wild type threonine to a glycine (hCPA1-T268G)	hCPA1-T268G was conjugated to ING-1 (antibody that binds to the tumor antigen Ep-Cam) or to Campath-1H (an antibody that binds to the T and B cell antigen CDw52) which acts on MTX-α-3-cyclobutylphenylalanine and MTX-α-3-cyclopentyltyrosine prodrugs releasing free methotrexate. It is found that ING-1:hCPA1-T268G conjugate produced excellent activation of the MTX prodrugs to kill HT-29 cells as efficiently as MTX itself	[247]
Mutant prolylendopeptidase (Glu289 → Gly)	Mutant human prolyl endopeptidase was chemically conjugated with L19 human antibody, a marker of angiogenesis which acts on the prodrug N-protected glycine-proline dipeptide doxorubicin or melphalan, i.e., Benzyloxycarbonyl) glycylprolyl melphalan producing free melphalan	[264]
Nitroreductase enzyme	It bioactivates CB 1954 (alkylating agent) much more rapidly than Walker DT diaphorase as a result it overcomes the intrinsic resistance of human cell lines towards CB 1954	[265]
Penicillin-V amidase (PVA)	Folate-PVA-^{125}I (PVA covalently labeled with three molecules of folic acid) which converts doxorubicin-*N*-*p*-hydroxy phenoxyacetamide prodrug (DPO) into its potent parent drug, doxorubicin, bind specifically to KB cells (FR-positive tumor cells) but not to A549 cells (FR-negative tumor cells) with higher clearance from the blood	[266]

Fifthly, prodrugs remains the fundamental and rate limiting component of ADEPT. The ratio of activity of therapeutically active form to the inactive form majorly determines the potency of the ADEPT in concern and this ratio usually ranges from 100 to 1,000 s. Classically it is anticipated that the dose response of the

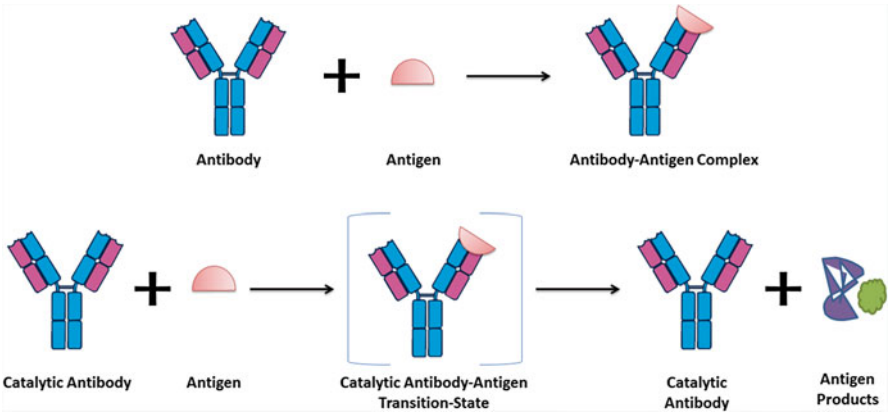

Fig. 2.14 Conceptual understanding of catalytic antibody

active drugs should be concentration dependent and should ideally have very low decay half-life so as to avoid any detrimental effects to the normal tissues from the drug that leaked into central compartment.

Recent trend in the field of ADEPT include design and developments in the field of two and three phase systems. Apart from activation of prodrug at tumor site, efforts are being made to rapidly clear the antibody–enzyme conjugates from the blood compartment in the view to avoid any possible toxicity to the normal tissues. The hypothesis has been classically tested in LS174T xenografted mice administered with monoclonal anti-carcinoembryonic antigen antibody fragment A5B7-F(ab′)$_2$ conjugated to a bacterial enzyme, carboxypeptidase G2 (CPG2) [254]. Among the tested approaches, the first comprised inactivator of CPG2, SB43, which was galactosylated and administered to clear out plasma antibody–enzyme conjugates mediated by carbohydrate receptor in liver. Interestingly, upon optimizing dose schedule, minimal compromise in therapeutic efficacy could be achieved. The second approach in contrast includes direct galactosylation of the antibody–enzyme conjugate in a manner such that binding affinity with the tumor antigen is altered. The said conjugate acts on receptor saturation mechanism and either rapidly bound to tumor tissues or cleared from the plasma cumulatively leading to almost 100-fold tumor to blood ratio of antibody–enzyme conjugates.

Furthermore, on similar line of ADEPT, Gene directed enzyme prodrug therapy (GDEPT) has also been widely explored for its potential in improving the tumor delivery of anticancer therapeutics. It is also referred to as suicide gene therapy (SGT), virus directed enzyme prodrug therapy (VDEPT), and gene prodrug activation therapy (GPAT). The principal difference herein is the activation of drug at intracellular level in contrast to ADEPT which releases the drug in extracellular matrices. The principal examples include activation of cyclophosphamide and ifosfamide using cytochrome p450, 5-fluorocytidine and 5-fluorouridine using cytosine deaminase, etc. Primarily, the tumor specific regulatory element and gene encoding enzyme is loaded in a suitable colloidal carrier system which upon reaching at

intracellular levels results in transgene expression and leads to execution of prodrug activation system, thereby imparting cell cytotoxicity [269].

2.5.3.5 Immunoliposomes

Liposomes represent an important class of nanocarriers with established applicability in a variety of disease conditions and difficult-to-deliver therapeutics. Though concomitant with widespread applicability, there also lie some negative points such as rapid clearance from the plasma process of opsonization of proteins on the surface of plain phospholipid vesicles. However, with the advent of PEGylation, the problem has been addressed to some extent and some quality products have made their way to clinical practice such as Doxil®. This stealth property of hydrophilic coatings enable liposomal drug delivery system to evade the reticuloendothelial systems and lead to preferential localization in the vicinity of extracellular tumor matrices by so-called enhanced permeation and retention effect [270]. However, in the purview of further increasing the therapeutic potential of these sophisticated colloidal drug delivery systems to tumor tissues, antibody targeting could be sought for. Structurally, these comprise stealth liposomes end functionalized with antibody, usually referred to as immunoliposomes [271]. Both whole antibody and antibody fractions specific to tumor antigens have been exhaustively used for exploring the potential of immunoliposomes for cancer therapy.

In the purview of formulation development, numerous methods have been proposed till date for linking antibodies to liposomal surface; however, post insertion technique remains the most viable and widely accepted [272]. In some instances, even the micelles of PEG linked antibodies have been incubated with marketed liposomal products to form immunoliposomes [273]. The classical chemical strategies include linking hydrazine group of PEG chains with oxidized carbohydrate group of oligosaccharide portion of antibody, maleimide group linked with thiolated antibodies, pyridyldithio propionate method, and biotin–avidin method [274]. Employing these chemistries numerous antigen targeted ligands have been linked to liposomes; however, the fruitful alteration in the various physicochemical properties of immunoliposomes and its optimization is still underway. Primarily, internalizing antibodies are employed for achieving significant appreciation in the therapeutic efficacy such as CD19 epitopes for B-cell lymphomas [275]. Figure 2.15 depicts various internalization mechanisms with liposomal drug delivery systems. Recent trends include combining immunoliposomes with endosome disruptive peptides for improved cytosolic delivery of therapeutics such as fusogenic peptide diINF-7 linked liposomes targeted to ovarian carcinoma [276].

The principal advantage associated with immunoliposomes is the very high drug payload as compared to any other available immunotherapies. Secondly, liposomal surface pose maximum capability to link the antibodies than classical immunotherapeutic approaches. This feature could be fruitfully exploited in employing monovalent antibody fragments, thereby eliminating the need of formation of scFv fragments by excessive antibody engineering to improve upon their valences and

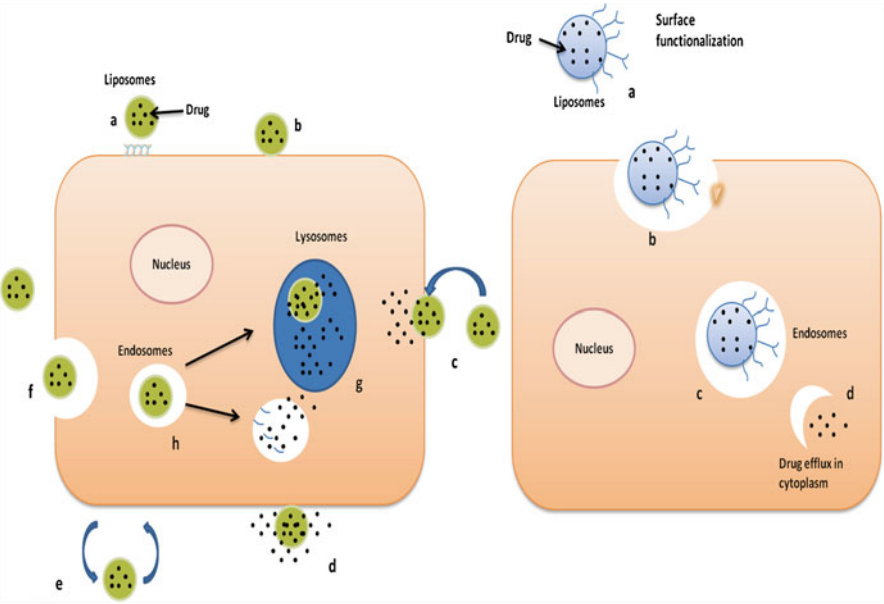

Fig. 2.15 Internalization of liposomal drug delivery systems. (A) Plain liposomes are entitled for either specific adsorption (**a**), nonspecific adsorption at cell surface (**b**) fusion with cell membrane (**c**), destabilized of liposomes upon adsorption at cell surface by internal components (**d**), direct or transfer-protein-mediated exchange of lipid component (**e**) and endocytosis (**f**). The endocytosed liposomes either fuses with lysosomes (**g**) or leads to endosomal escape and drug delivery to cytoplasm (**h**). (B) Surface functionalization of the liposomes with appropriate ligands can be performed (**a**) to interact with target receptors (**b**), followed by endocytosis (**c**), and drug release (**d**)

hence binding affinity [271]. The concept of immunoliposomes has emerged since old times and in numerous instances, potential has been established. Table 2.10 lists various approaches for immunoliposomes based drug delivery for efficient management of cancer [277].

2.5.3.6 Immunopolymers

Blending polymer engineering with antibody engineering is probably the most recent era of immunotherapy. Polymer based drug delivery is widely accepted as one stop solution for almost all kinds of therapeutics ranging from small molecules, nucleic acid, proteins, peptides, hormones, etc. The barriers of physicochemical properties of said therapeutics can be efficiently circumvented by designing apt polymeric drug delivery system. With the advent of antibody directed polymers, plethora of chemically and functionally diverse synthetic yet biomimetic systems have been explored in last couple of decades [294]. The principal advantages associated with antibody conjugation with polymers includes significant appreciation in solubility, immunocompatibility, favorable pharmacokinetics, improved stability,

Table 2.10 List of immunoliposomes based drug delivery system for cancer immunotherapy

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Anti-EGFR (epidermal growth factor receptor) Fab	Adriamycin and ribonucleotide Reductase M2 siRNA	EGFR positive hepatocellular carcinoma(HCC)	Targeted LPD (liposome–polycation–DNA complex) conjugated with anti-EGFR (epidermal growth factor receptor) Fab′ co-delivering adriamycin (ADR) and ribonucleotide reductase M2 (RRM2) siRNA (ADR-RRM2-TLPD) was prepared with enhanced therapeutic effects	[278]
Anti-EGFR-Fab	siRNA	MDA-MB-231 breast cancer cells	TLPD-FCC (Targeted liposome–polycation–DNA complex conjugated with anti-EGFR Fab′ by conventional conjugation) showed significantly enhanced binding affinity and luciferase gene silencing activity by ~20 % in EGFR	[279]
Anti-EGFR-Fab	siRNA	SMMC-7721 hepatocellular carcinoma cells	Two PEG derivative linkers (DSPE-PEG-COOH and DSPE-PEG-MAL) were used to develop immunoliposomes. Immunoliposomes derived from DSPE-PEG-MAL (TLPD-FPM) shown to have significantly greater cellular uptake and up to threefold higher luciferase gene silencing efficiency than that of TLPD-FPC	[280]

(continued)

Table 2.10 (continued)

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Anti-EGFR and anti-HER2	Topotecan (TPT)	HER2 overexpressing human breast cancer (BT474)	Stabilization of topotecan in nanoliposomes significantly improves the targetability and pharmacokinetic profile of topotecan	[281]
Anti-HB-EGF	Doxorubicin	HB-EGF positive breast cancer	Results showed selective binding and uptake of the immunoliposomes in HB-EGF-expressing cells	[282]
Antinucleosome monoclonal antibody 2C5	Doxorubicin	Nucleosome	Significant reduction in tumor growth and enhanced therapeutic efficacy of the drug in both drug resistant and drug sensitive mice was obtained	[283]
Anti-VEGFR2	Doxorubicin	HT-29 human colon cancer/MMTV-PyMT mouse model of breast cancer	Histopathological and molecular analysis revealed strong antiangiogenic effect of anti-VEGFR2-ILs-dox (Anti-VEGFR2-targeted immunoliposomes (ILs) loaded with doxorubicin)	[284]
Anti-RON antibody Zt/c9	Doxorubicin	CD24+, CD44+, ESA + (triple positive) pancreatic L3.6pl cancer cells	Anti-RON antibody Zt/c9-directing doxorubicin-immunoliposomes (Zt/c9-Dox-IL) was developed which specifically interacted with CSCs $^{+24/44/ESA}$	[285]
Anti-RON antibody Zt/g4	Doxorubicin	Hypoxic colon HCT116 and SW620 cells	Zt/g4-Dox-IL was found to be effective in killing hypoxic HCT116 and SW620 cells with reduced IC 50 values compared to Dox and pegylated-liposomal Dox	[286]

(continued)

Table 2.10 (continued)

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Cetuximab (α-hEGFR)	–	Glioblastoma multiforme	In vitro studies revealed significantly higher binding of α-hEGFR-ILs (PEGylated immunoliposomes conjugated with anti-human epidermal growth factor receptor (EGFR) antibodies) when compared with liposomes conjugated with isotypic nonimmune Ig resulting in enhanced uptake and accumulation of liposomes	[287]
Cetuximab	Doxorubicin	Human ovarian adenocarcinoma (SKOV3, SKOV3.i.p.1) cells	Showed efficient and specific receptor-mediated binding to ovarian carcinoma cells	[288]
IGFI-R antagonistic antibody (1H7)	Doxorubicin	Neuroendocrine tumors of the gastroenteropancreatic system (GEP-NETs)	Anti-IGFI-R immunoliposomes displayed specific tumor cell and internalization in human neuroendocrine tumor cells in vitro and superior antitumor efficacy in vivo	[289]
mAb 2C5	Doxorubicin	B16-F10, HeLa, and MCF-7 cell lines	Multifunctional immunoliposomal nanocarrier with pH-sensitive PEG-PE component, TATp and the cancer cell specific mAb 2C5 showed enhanced cytotoxicity and internalization by cancer cells	[290]
Trastuzumab	Bleomycin	HER2 positive human breast cancer	Immunoliposomes showed enhanced cytotoxicity towards HER2 positive MCF-7/Her18 cells and also affect trastuzumab-resistant MDA-453 cell	[291]

(continued)

Table 2.10 (continued)

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Trastuzumab	Curcumin and resveratrol	HER2 positive human breast cancer	Significant increase in the antiproliferative effects of curcumin and resveratrol in HER2 positive human breast cancer cells as a result of enhanced uptake of curcumin and resveratrol at intracellular level	[292]
Trastuzumab	Docetaxel	Her2/neu positive gastric tumor	Docetaxel-loaded immuno (trastuzumab) liposomes (IDL) showed a significantly higher distribution of docetaxel in the N87 xenograft tumor tissues and superior antitumor efficacy	[293]

relatively higher cellular uptake, reduced aggregation, relatively higher antibody payload, feasibility to modulate intracellular trafficking, etc. Recent advances in the field of recombinant technology has enable the use of a variety of products such as isolated antibodies and their fragments including humanized, tumor biomarkers, structural components of microbes, natural and synthetic sources of immunostimulators.

Immunopolymers primarily comprise antibody (either whole antibody or antibody fragments) linked to polymers (may be functionalized for specific applications). Recent advances in the field of antibody engineering enable the de novo design and development of antibody fragments for a variety of end applications [295]. These antibody fragments when linked to polymeric counterparts avail additional advantage of relatively higher binding density owing to smaller size [296]. PEGylation of antibody fragments has been explored to a greater extent in improving the circulation half-life, per se. A variety of approaches employed for linking these antibody fragments to polymer backbone include thiol modifications, linking via sugar portions, fusion proteins, etc. Generally, either N- or C- terminal end of the antibody fragments is opted for modifications; however, in some cases such as in scFvs inter-domain peptide linker may be sought for without any compromise in binding affinity [297]. Recently, novel functionalities in the scFvs could be added employing a variety of protein and chemical engineering approaches such as tagging with hexahistidine or streptavidin for site specific conjugation or delivery [298]. Similarly, C-terminal cysteine modification has also been greatly explored and could lead to ~100-fold appreciation in circulation half-life [299]. Furthermore, PEGylation of scFvs also render the resulting immunopolymers resistant to proteolytic enzymes [300]. Owing to these functionalities, PEGylated antibody fragments have been widely explored from tumor targeting perspectives. In a representative

study, PEGylated di(Fab′) exhibited significantly higher antitumor efficacy as compared to PEGylated IgG and the latter being comparable to that of plain IgG in xenograft tumor bearing thymic mice [301]. Similar results were also noted in case of PEGylated anti-CEA $F(ab')_2$ exhibiting significant increase in circulation half-life and tumor accumulation [302]. In separate set of experiments effect of molecular weight of PEG was also studied and it was found that higher molecular weights in the order of 25 kD tend to localize equally within normal tissues also in contrast to that of low molecular weights (~5 kD) [303]. In an interesting study, ^{111}In-cysteinyl-DOTA-PEG3400-diabody conjugate and ^{125}I-PEG3400-diabody were explored for its potential in imaging liver metastasis in a nude mouse xenograft model (Fig. 2.16) [304].

Apart from PEGylated antibodies, stimuli responsive polymers represent yet another area where antibodies are being explored to a greater extent. Classically, these stimuli responsive polymers are sensitive to pH, temperature, presence of

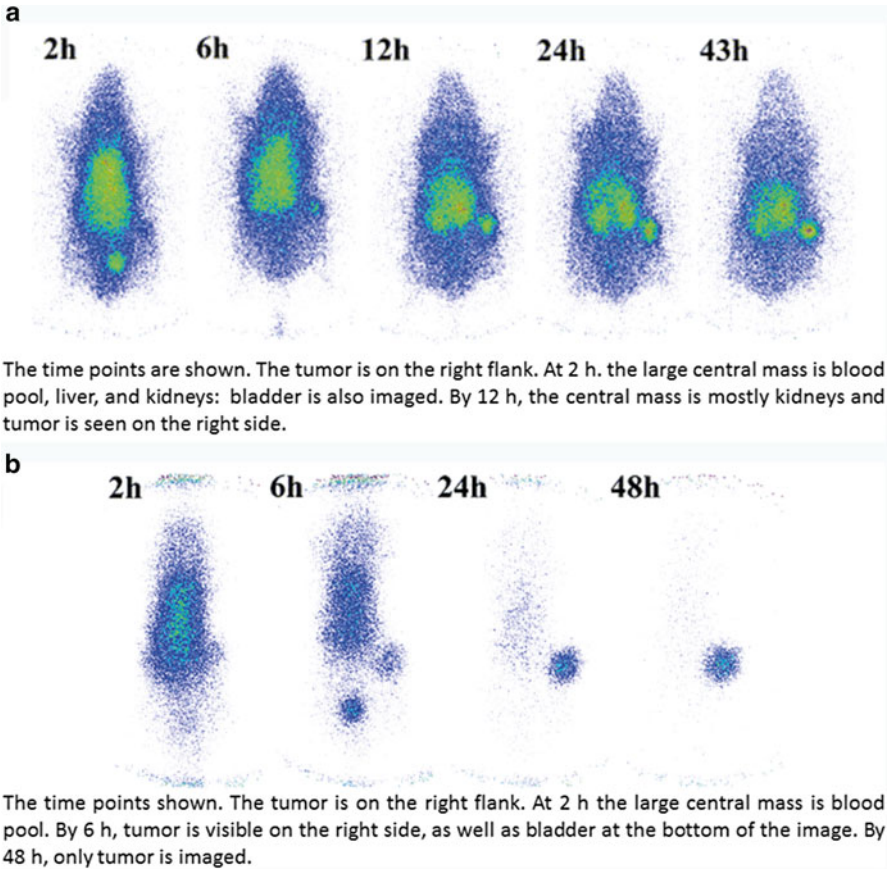

Fig. 2.16 Radioimmunoimaging of (**a**) ^{111}In-cys-DOTA-PEG3400-diabody and (**b**) ^{125}I-PEG3400-diabody in nude mice bearing LS174T xenografts. Reproduced from ref. [304]

small molecules such as amino acids, external energies such as electrical and magnetic, etc. [294]. In this domain, *N*- (2 hydroxy propyl) methacrylamide (HPMA) has been exhaustively explored. Significant increase in the cell cytotoxicity of Fab′ was noted against ovarian carcinoma when co-polymerized with HPMA [305]. On similar line of action, galactosylated HPMA conjugate comprising doxorubicin with Gly-Phe-Leu-Gly spacer revealed great clinical potential with notable responses in hepatoma patients [306]. Mechanistic studies with anti-Thy1.2 targeted or CD71 targeted HPMA polymers further revealed preferential nuclear localization tendencies intracellularly [307]. Recent advances in the field of polymer drug conjugates include its combination with ADEPT and the resulting therapy is referred to as polymer directed enzyme prodrug therapy (PDEPT) where combination of polymeric prodrug and polymer enzyme conjugate is employed to impart cytotoxicity at the site of action. PK1-HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin conjugate in combination with HPMA copolymer-cathepsin B led to 4.2-fold higher tumor accumulation in B16F10 tumor bearing mice as compared to plain free enzyme [308]. Table 2.11 lists immunopolymers employed for improving the deliverability of antibodies.

2.5.4 Targeting Multidrug Resistant Tumors

The principal problem associated with the advanced anticancer therapeutics, i.e., molecular targeted therapies and immunotherapy are increased chances of resistance and inter-, intra-tumor variability, often leading to poor therapeutic responses. Broadly, two factors have been considered responsible for multidrug resistance, viz., cellular and physiological factors [319]. The former includes a variety of genetic alterations at cellular levels such as efflux transporters, whereas the latter is more focused on the physicochemical changes at tissue levels such as pH, extracellular interactions and peculiar tumor microenvironment (Fig. 2.17). Based on these factors, efflux of the bioactives is regarded as most common mechanism of drug resistance in cancer therapeutics [320]. A variety of approaches could be employed to counterfeit drug efflux systems and these include pharmacologically active P-gp inhibitors, functional excipients such as natural polymers, surfactants, lipids, cyclodextrins, polyethylene glycol and derivatives, thiolated polymers, etc. [16].

Furthermore, in purview of increasing therapeutic responses in cancer chemotherapy and sensitize the multidrug-resistant tumors, nanotechnology seems to be most efficient approach. Recent advances in the field of nanocarrier based approaches have paved the way to efficiently deliver therapeutics to multidrug resistant cancer therapy and includes polymeric nanoparticles, lipid nanocarriers, dendrimers, carbon nanotubes and inorganic nanocarriers, to name a few. The principal focus of employing nanocarriers relies on improving the interactions with target cells, enhanced internalization mechanisms, tumor specific biodistribution pattern, availing benefits of “click chemistry,” reducing the nonspecific binding, tailoring the ligand properties such as choice of ligand and its density, charge, orientation, etc. [322].

Table 2.11 List of immunopolymers employed for improving the deliverability of antibodies

Antibody	Polymer	Outcomes	Ref.
Anti-CD20 monoclonal antibody	HPMA	Cytostatic activity of the anti-CD20 monoclonal Ab-targeted conjugates tested on several CD20-positive or negative human and mouse cancer cell lines confirmed considerable targeting capacity of the monoclonal Ab after its binding to the polymer carrier	[309]
Anti-EGF receptor antibody	DSPE-PEG lipid polymer complex	In vivo accumulation of PLNP-Mal-EGFR was found to be higher than that of nontargeted nanoparticles in SMMC-7721 HCC cells overexpressing EGFR with enhanced antitumor activity against HCC compared with nontargeted nanoparticles and free adriamycin	[310]
Anti-H*ER2*-affibody-anti-DTPA-Fab complexes (BAAC), anti-DTPA-Fab	Polyglutamic acid	There was no total body weight (TBW) loss at three times the doxorubicin equivalent maximum tolerated dose (MTD) with D-Dox-PGA. Therapeutic efficacy was equivalent in mice pre-targeted with BAAC/ targeted with D-Dox-PGA to mice treated only with doxorubicin	[311]
Anti-PSMA antibody	HPMA	Rate of endocytosis of P-anti-PSMA was much faster than that of control HPMA copolymer conjugates containing nonspecific IgGvia clathrin-mediated endocytosis, macropinocytosis, and clathrin-, caveolae-independent endocytosis	[312]
Catalytic antibody 38C2	HPMA	Catalytic antibody–HPMA copolymer conjugate was evaluated in vitro for its ability to activate an etoposide prodrug and it was found that the inhibition using the prodrug and the conjugate was almost identical to inhibition by the free antibody and the prodrug	[313]
HD39 monoclonal antibody	Poly(propylacrylic acid) (PPAA)	Subcellular fractionation studies of HD39/ SA-PPAA conjugates showed 89 % of HD39/ SA was associated with endosomes (Rab5+) and lysosomes (Lamp2+), while 45 % of HD39/ SA-PPAA was translocated to the cytosol (lactate dehydrogenase+) which demonstrate the endosomal releasing properties of PPAA with antibody–polymer conjugates	[314]
Monoclonal anti-RAGE and polyclonal human Ig (huIgG)	Poly(*N*-(2-hydroxypropyl)-methacrylamide) (poly-HPMA)	Antibody polymer conjugate, two different model antibodies, monoclonal anti-RAGE and polyclonal human Ig (huIgG) antibodies, were attached to maleimide functionalized poly(N-(2-hydroxypropyl)-methacrylamide) (poly-HPMA) through reversible addition -fragmentation chain transfer (RAFT) polymerization of pentafluorophenyl methacrylate via the intermediate step of an activated ester polymer was developed with preserved affinity	[315]

(continued)

Table 2.11 (continued)

Antibody	Polymer	Outcomes	Ref.
HPMA	Polyclonal and monoclonal anti-Thy 1.2 or anti-Ia^k antibody	Daunomycin toxicity of daunomycin-antibody-copolymer conjugate against hematopoietic precursors in bone marrow colony forming unit spleen was found to be decreased up to 80-fold and with no significant irritation of Kupffer cells in liver	[316]
OV-TL16 antibody	Pegylated polyethylenimine (PEG-PEI)	Sixfold higher degree of binding of PEG-PEI-Fab'/DNA complexes to OA3 positive human ovarian carcinoma cell lines compared to unmodified PEG-PEI/DNA complexes and up to 80-fold increase in luciferase reporter gene expression compared to PEG-PEI	[317]
Polyclonal rabbit anti-mouse thymocyte globulin (ATG)	Poly(ethylene glycol) (PEG)	Antibody polymer drug conjugates exhibited significant antitumor efficiency against murine T-cell EL 4 lymphoma in vivo	[318]

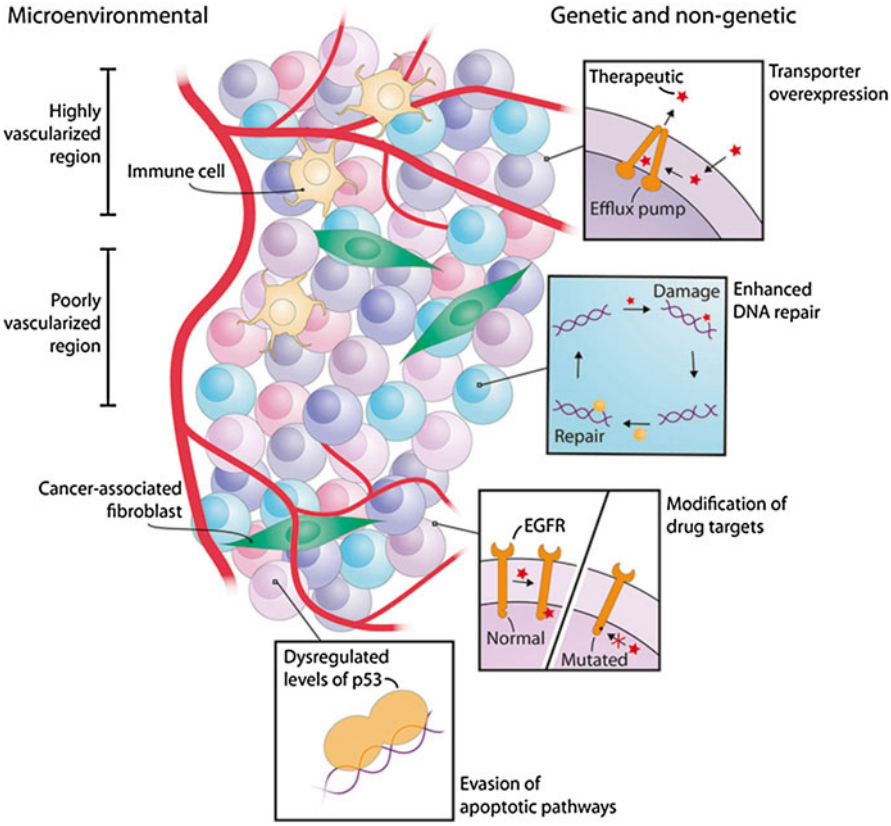

Fig. 2.17 Factors influencing tumor heterogeneity and drug resistance. Genetic, nongenetic, and microenvironmental factors give rise to tumor heterogeneity, which significantly influences the drug sensitivity of cancer cells through an array of cellular mechanisms, such as transporter overexpression. Reproduced from ref. [321]

2.6 Conclusion and Future Prospects

Recent advances in the field of tumor targeting focus on the design and development of highly sophisticated molecules with high tumor specificity. Molecular targeted therapies are rapidly changing its paradigm towards tumor specific antigen and have reached quite near to the original concept of *magic bullet*. Furthermore, in combination with antiangiogenics, immunotherapy and nanotechnology based approaches, the efficient management of even multidrug resistant tumors is also quite possible and several studies are currently under exhaustive clinical trials. The future work in the field of cancer therapy includes clearing the clinical trial pipeline into approved products followed by dedicated cancer prevention programs such as cancer vaccines.

References

1. International Agency for Research on Cancer (IARC), World Heatlth Organization (WHO) (2012)Cancer fact sheets. In: Globocan 2012: estimated cancer incidence, mortalitity and prevalence worldwide in 2012. International Agency for Research on Cancer (IARC), World Heatlth Organization (WHO), Lyon, France
2. Lippman SM, Hong WK (2002) Cancer prevention science and practice. Cancer Res 62:5119–5125, PMID: 12234971
3. Ghose T (2002) The current status of tumor targeting. In: Page M (ed) Tumor targeting in cancer therapy. Springer Science, New York, NY, pp 3–78
4. Poste G, Kirsh R (1983) Site–specific (targeted) drug delivery in cancer therapy. Nat Biotechnol 1:869–878. doi:10.1038/nbt1283-869
5. Parveen S, Sahoo SK (2008) Polymeric nanoparticles for cancer therapy. J Drug Target 16:108–123. doi:10.1080/10611860701794353
6. Dewhirst MW, Kimura H, Rehmus SW, Braun RD, Papahadjopoulos D, Hong K, Secomb TW (1996) Microvascular studies on the origins of perfusion-limited hypoxia. Br J Cancer 27(Suppl):S247–S251, PMID 8763890
7. Cairns R, Papandreou I, Denko N (2006) Overcoming physiologic barriers to cancer treatment by molecularly targeting the tumor microenvironment. Mol Cancer Res 4:61–70. doi:10.1158/1541-7786.MCR-06-0002
8. Heldin CH, Rubin K, Pietras K, Ostman A (2004) High interstitial fluid pressure – an obstacle in cancer therapy. Nat Rev Cancer 4:806–813. doi:10.1038/nrc1456
9. van den Berg AP, Wike-Hooley JL, van den Berg-Blok AE, van der Zee J, Reinhold HS (1982) Tumour pH in human mammary carcinoma. Eur J Cancer Clin Oncol 18:457–462. doi:10.1016/0277-5379(82)90114-6
10. Tannock IF, Rotin D (1989) Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 49:4373–4384, PMID: 2545340
11. Weinhouse S (1976) The Warburg hypothesis fifty years later. Z Krebsforsch Klin Onkol Cancer Res Clin Oncol 87:115–126, PMID: 136820
12. Yamagata M, Hasuda K, Stamato T, Tannock IF (1998) The contribution of lactic acid to acidification of tumours: studies of variant cells lacking lactate dehydrogenase. Br J Cancer 77:1726–1731. doi:10.1038/bjc.1998.289
13. Izumi H, Torigoe T, Ishiguchi H, Uramoto H, Yoshida Y, Tanabe M, Ise T, Murakami T, Yoshida T, Nomoto M, Kohno K (2003) Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. Cancer Treat Rev 29:541–549. doi:10.1016/S0305-7372(03)00106-3

14. Griffiths JR, McIntyre DJ, Howe FA, Stubbs M (2001) Why are cancers acidic? A carrier-mediated diffusion model for H+transport in the interstitial fluid. Novartis Found Symp 240:46–62, discussion 62–47, 152–153; PMID: 11727936
15. Narang AS, Varia S (2011) Role of tumor vascular architecture in drug delivery. Adv Drug Deliv Rev 63:640–658. doi:10.1016/j.addr.2011.04.002
16. Thanki K, Gangwal RP, Sangamwar AT, Jain S (2013) Oral delivery of anticancer drugs: challenges and opportunities. J Control Release 170:15–40. doi:10.1016/j.jconrel.2013.04.020
17. Weissig V, D'Souza GG (2010) Organelle-specific pharmaceutical nanotechnology. Wiley, Hoboken, NJ
18. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46:6387–6392, PMID: 2946403
19. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK (1998) Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proc Natl Acad Sci U S A 95:4607–4612, PMID: 9539785
20. Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E, Jain RK (2004) Pathology: cancer cells compress intratumour vessels. Nature 427:695. doi:10.1038/427695a
21. Rabanel JM, Aoun V, Elkin I, Mokhtar M, Hildgen P (2012) Drug-loaded nanocarriers: passive targeting and crossing of biological barriers. Curr Med Chem 19:3070–3102. doi:10.2174/092986712800784702
22. Nag OK, Awasthi V (2013) Surface engineering of liposomes for stealth behavior. Pharmaceutics 5:542–569. doi:10.3390/pharmaceutics5040542
23. Salmaso S, Caliceti P (2013) Stealth properties to improve therapeutic efficacy of drug nanocarriers. J Drug Deliv 2013:374252. doi:10.1155/2013/374252
24. Allen TM, Hansen C, Rutledge J (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim Biophys Acta 981:27–35. doi:10.1016/0005-2736(89)90078-3
25. Gabizon A, Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 85:6949–6953, PMID: 3413128
26. Immordino ML, Dosio F, Cattel L (2006) Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomedicine 1:297–315, PMID: 17717971
27. Tirosh O, Barenholz Y, Katzhendler J, Priev A (1998) Hydration of polyethylene glycol-grafted liposomes. Biophys J 74:1371–1379. doi:10.1016/S0006-3495(98)77849-X
28. Lehtonen JY, Kinnunen PK (1995) Poly(ethylene glycol)-induced and temperature-dependent phase separation in fluid binary phospholipid membranes. Biophys J 68:525–535. doi:10.1016/S0006-3495(95)80214-6
29. Szebeni J (2005) Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. Toxicology 216:106–121. doi:10.1016/j.tox.2005.07.023
30. Yang Q, Ma Y, Zhao Y, She Z, Wang L, Li J, Wang C, Deng Y (2013) Accelerated drug release and clearance of PEGylated epirubicin liposomes following repeated injections: a new challenge for sequential low-dose chemotherapy. Int J Nanomedicine 8:1257–1268. doi:10.2147/IJN.S41701
31. Herold DA, Keil K, Bruns DE (1989) Oxidation of polyethylene glycols by alcohol dehydrogenase. Biochem Pharmacol 38:73–76. doi:10.1016/0006-2952(89)90151-2
32. Veronese FM, Pasut G (2005) PEGylation, successful approach to drug delivery. Drug Discov Today 10:1451–1458. doi:10.1016/S1359-6446(05)03575-0
33. Allen TM (2002) Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2:750–763. doi:10.1038/nrc903
34. Gradishar WJ (2006) Albumin-bound paclitaxel: a next-generation taxane. Expert Opin Pharmacother 7:1041–1053. doi:10.1517/14656566.7.8.1041
35. John TA, Vogel SM, Tiruppathi C, Malik AB, Minshall RD (2003) Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer. Am J Physiol Lung Cell Mol Physiol 284:L187–196. doi:10.1152/ajplung.00152.2002

36. Minshall RD, Tiruppathi C, Vogel SM, Malik AB (2002) Vesicle formation and trafficking in endothelial cells and regulation of endothelial barrier function. Histochem Cell Biol 117:105–112. doi:10.1007/s00418-001-0367-x
37. Kumagai AK, Eisenberg JB, Pardridge WM (1987) Absorptive-mediated endocytosis of cationized albumin and a beta-endorphin-cationized albumin chimeric peptide by isolated brain capillaries. Model system of blood-brain barrier transport. J Biol Chem 262:15214–15219, PMID: 2959663
38. Desai N, Trieu V, Yao Z, Louie L, Ci S, Yang A, Tao C, De T, Beals B, Dykes D, Noker P, Yao R, Labao E, Hawkins M, Soon-Shiong P (2006) Increased antitumor activity, intratumor paclitaxel concentrations, and endothelial cell transport of cremophor-free, albumin-bound paclitaxel, ABI-007, compared with cremophor-based paclitaxel. Clin Cancer Res 12:1317–1324. doi:10.1158/1078-0432.CCR-05-1634
39. Das M, Mohanty C, Sahoo SK (2009) Ligand-based targeted therapy for cancer tissue. Expert Opin Drug Deliv 6:285–304. doi:10.1517/17425240902780166
40. Vasir JK, Labhasetwar V (2005) Targeted drug delivery in cancer therapy. Technol Cancer Res Treat 4:363–374, PMID: 16029056
41. Jain S, Mathur R, Das M, Swarnakar NK, Mishra AK (2011) Synthesis, pharmacoscintigraphic evaluation and antitumor efficacy of methotrexate-loaded, folate-conjugated, stealth albumin nanoparticles. Nanomedicine (Lond) 6:1733–1754. doi:10.2217/nnm.11.53
42. Das M, Bandyopadhyay D, Mishra D, Datir S, Dhak P, Jain S, Maiti TK, Basak A, Pramanik P (2011) "Clickable", trifunctional magnetite nanoparticles and their chemoselective biofunctionalization. Bioconjug Chem 22:1181–1193. doi:10.1021/bc2000484
43. Das M, Datir SR, Singh RP, Jain S (2013) Augmented anticancer activity of a targeted, intracellularly activatable, theranostic nanomedicine based on fluorescent and radiolabeled, methotrexate-folic acid-multiwalled carbon nanotube conjugate. Mol Pharm 10:2543–2557. doi:10.1021/mp300701e
44. Lu YJ, Wei KC, Ma CC, Yang SY, Chen JP (2012) Dual targeted delivery of doxorubicin to cancer cells using folate-conjugated magnetic multi-walled carbon nanotubes. Colloids Surf B: Biointerfaces 89:1–9. doi:10.1016/j.colsurfb.2011.08.001
45. Li L, Yang Q, Zhou Z, Zhong J, Huang Y (2014) Doxorubicin-loaded, charge reversible, folate modified HPMA copolymer conjugates for active cancer cell targeting. Biomaterials 35:5171–5187. doi:10.1016/j.biomaterials.2014.03.027
46. Corbin IR, Ng KK, Ding L, Jurisicova A, Zheng G (2013) Near-infrared fluorescent imaging of metastatic ovarian cancer using folate receptor-targeted high-density lipoprotein nanocarriers. Nanomedicine 8:875–890. doi:10.2217/nnm.12.137
47. Hong G, Yuan R, Liang B, Shen J, Yang X, Shuai X (2008) Folate-functionalized polymeric micelle as hepatic carcinoma-targeted, MRI-ultrasensitive delivery system of antitumor drugs. Biomed Microdevices 10:693–700. doi:10.1007/s10544-008-9180-9
48. Tyagi N, Ghosh PC (2011) Folate receptor mediated targeted delivery of ricin entrapped into sterically stabilized liposomes to human epidermoid carcinoma (KB) cells: effect of monensin intercalated into folate-tagged liposomes. Eur J Pharm Sci 43:343–353. doi:10.1016/j.ejps.2011.05.010
49. Lale SV, GA R, Aravind A, Kumar DS, Koul V (2014) AS1411 Aptamer and folic acid functionalized pH-responsive ATRP fabricated pPEGMA-PCL-pPEGMA polymeric nanoparticles for targeted drug delivery in cancer therapy. Biomacromolecules 15:1737–1752. doi:10.1021/bm5001263
50. El-Gogary RI, Rubio N, Wang JT, Al-Jamal WT, Bourgognon M, Kafa H, Naeem M, Klippstein R, Abbate V, Leroux F, Bals S, Van Tendeloo G, Kamel AO, Awad GA, Mortada ND, Al-Jamal KT (2014) Polyethylene glycol conjugated polymeric nanocapsules for targeted delivery of quercetin to folate-expressing cancer cells in vitro and in vivo. ACS Nano 8:1384–1401. doi:10.1021/nn405155b
51. Tavassolian F, Kamalinia G, Rouhani H, Amini M, Ostad SN, Khoshayand MR, Atyabi F, Tehrani MR, Dinarvand R (2014) Targeted poly (L-gamma-glutamyl glutamine) nanoparticles of docetaxel against folate over-expressed breast cancer cells. Int J Pharm 467:123–138. doi:10.1016/j.ijpharm.2014.03.033

52. Nair BP, Vaikkath D, Nair PD (2014) Polyhedral oligomeric silsesquioxane-F68 hybrid vesicles for folate receptor targeted anti-cancer drug delivery. Langmuir 30:340–347. doi:10.1021/la4036997
53. Chen YC, Chiang CF, Chen LF, Liang PC, Hsieh WY, Lin WL (2014) Polymersomes conjugated with des-octanoyl ghrelin and folate as a BBB-penetrating cancer cell-targeting delivery system. Biomaterials 35:4066–4081. doi:10.1016/j.biomaterials.2014.01.042
54. Dowlati A, Loo M, Bury T, Fillet G, Beguin Y (1997) Soluble and cell-associated transferrin receptor in lung cancer. Br J Cancer 75:1802–1806. doi:10.1038/bjc.1997.307
55. Gosk S, Vermehren C, Storm G, Moos T (2004) Targeting anti-transferrin receptor antibody (OX26) and OX26-conjugated liposomes to brain capillary endothelial cells using in situ perfusion. J Cereb Blood Flow Metab 24:1193–1204. doi:10.1097/01.WCB.0000135592.28823.47
56. Sahoo SK, Labhasetwar V (2005) Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. Mol Pharm 2:373–383. doi:10.1021/mp050032z
57. Sahoo SK, Ma W, Labhasetwar V (2004) Efficacy of transferrin-conjugated paclitaxel-loaded nanoparticles in a murine model of prostate cancer. Int J Cancer 112:335–340. doi:10.1002/ijc.20405
58. Ferris DP, Lu J, Gothard C, Yanes R, Thomas CR, Olsen JC, Stoddart JF, Tamanoi F, Zink JI (2011) Synthesis of biomolecule-modified mesoporous silica nanoparticles for targeted hydrophobic drug delivery to cancer cells. Small 7:1816–1826. doi:10.1002/smll.201002300
59. Kim TH, Jo YG, Jiang HH, Lim SM, Youn YS, Lee S, Chen X, Byun Y, Lee KC (2012) PEG-transferrin conjugated TRAIL (TNF-related apoptosis-inducing ligand) for therapeutic tumor targeting. J Control Release 162:422–428. doi:10.1016/j.jconrel.2012.07.021
60. Gaspar MM, Radomska A, Gobbo OL, Bakowsky U, Radomski MW, Ehrhardt C (2012) Targeted delivery of transferrin-conjugated liposomes to an orthotopic model of lung cancer in nude rats. J Aerosol Med Pulm Drug Deliv 25:310–318. doi:10.1089/jamp.2011.0928
61. Liu G, Shen H, Mao J, Zhang L, Jiang Z, Sun T, Lan Q, Zhang Z (2013) Transferrin modified graphene oxide for glioma-targeted drug delivery: in vitro and in vivo evaluations. ACS Appl Mater Interfaces 5:6909–6914. doi:10.1021/am402128s
62. Neves S, Faneca H, Bertin S, Konopka K, Duzgunes N, Pierrefite-Carle V, Simoes S, Pedroso de Lima MC (2009) Transferrin lipoplex-mediated suicide gene therapy of oral squamous cell carcinoma in an immunocompetent murine model and mechanisms involved in the anti-tumoral response. Cancer Gene Ther 16:91–101. doi:10.1038/cgt.2008.60
63. Vaidya B, Vyas SP (2012) Transferrin coupled vesicular system for intracellular drug delivery for the treatment of cancer: development and characterization. J Drug Target 20:372–380. doi:10.3109/1061186X.2012.662687
64. Abouzeid AH, Patel NR, Sarisozen C, Torchilin VP (2014) Transferrin-targeted polymeric micelles co-loaded with curcumin and paclitaxel: efficient killing of paclitaxel-resistant cancer cells. Pharm Res 1-8. doi: 10.1007/s11095-013-1295-x
65. Yhee JY, Lee SJ, Lee S, Song S, Min HS, Kang SW, Son S, Jeong SY, Kwon IC, Kim SH, Kim K (2013) Tumor-targeting transferrin nanoparticles for systemic polymerized siRNA delivery in tumor-bearing mice. Bioconjug Chem 24:1850–1860. doi:10.1021/bc400226b
66. Huang Y, He L, Liu W, Fan C, Zheng W, Wong Y-S, Chen T (2013) Selective cellular uptake and induction of apoptosis of cancer-targeted selenium nanoparticles. Biomaterials 34:7106–7116. doi:10.1016/j.biomaterials.2013.04.067
67. Bies C, Lehr CM, Woodley JF (2004) Lectin-mediated drug targeting: history and applications. Adv Drug Deliv Rev 56:425–435. doi:10.1016/j.addr.2003.10.030
68. Baenziger JU, Maynard Y (1980) Human hepatic lectin. Physiochemical properties and specificity. J Biol Chem 255:4607–4613, PMID: 7372599
69. Duncan R (2003) The dawning era of polymer therapeutics. Nat Rev Drug Discov 2:347–360. doi:10.1038/nrd1088
70. Yu HY, Lin JH (2000) Intracellular delivery of membrane-impermeable hydrophilic molecules to a hepatoblastoma cell line by asialoglycoprotein-labeled liposomes. J Formos Med Assoc 99:936–941, PMID: 11155748

71. Dubey PK, Mishra V, Jain S, Mahor S, Vyas SP (2004) Liposomes modified with cyclic RGD peptide for tumor targeting. J Drug Target 12:257–264. doi:10.1080/10611860410001728040
72. Aina OH, Sroka TC, Chen ML, Lam KS (2002) Therapeutic cancer targeting peptides. Biopolymers 66:184–199. doi:10.1002/bip.10257
73. Torchilin VP (2000) Drug targeting. Eur J Pharm Sci 11(Suppl 2):S81–91. doi:10.1016/S0928-0987(00)00166-4
74. Husseini GA, Diaz de la Rosa MA, Gabuji T, Zeng Y, Christensen DA, Pitt WG (2007) Release of doxorubicin from unstabilized and stabilized micelles under the action of ultrasound. J Nanosci Nanotechnol 7:1028–1033. doi:10.1166/jnn.2007.218
75. Knights CD, Pestell RG (2008) The cell cycle: therapeutic targeting of cell cycle regulatory components and effector pathways in cancer. In: Kaufman HL, Wadler S, Antman K (eds) Molecular targeting in oncology. Humana, Totowa, NJ
76. Liu MC, Marshall JL, Pestell RG (2004) Novel strategies in cancer therapeutics: targeting enzymes involved in cell cycle regulation and cellular proliferation. Curr Cancer Drug Targets 4:403–424. doi:10.2174/1568009043332907
77. Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K, Miranda M, Krig S, Garbe J, Stampfer M, Yaswen P, Gray JW, Lockett SJ (2004) In situ analyses of genome instability in breast cancer. Nat Genet 36:984–988. doi:10.1038/ng1409
78. Deshpande A, Sicinski P, Hinds PW (2005) Cyclins and cdks in development and cancer: a perspective. Oncogene 24:2909–2915. doi:10.1038/sj.onc.1208618
79. Hedenfalk I, Duggan D, Chen Y, Radmacher M, Bittner M, Simon R, Meltzer P, Gusterson B, Esteller M, Kallioniemi OP, Wilfond B, Borg A, Trent J, Raffeld M, Yakhini Z, Ben-Dor A, Dougherty E, Kononen J, Bubendorf L, Fehrle W, Pittaluga S, Gruvberger S, Loman N, Johannsson O, Olsson H, Sauter G (2001) Gene-expression profiles in hereditary breast cancer. N Engl J Med 344:539–548. doi:10.1056/NEJM200102223440801
80. Dobbelstein M, Moll U (2014) Targeting tumour-supportive cellular machineries in anticancer drug development. Nat Rev Drug Discov 13:179–196. doi:10.1038/nrd4201
81. Armstrong GT, Liu W, Leisenring W, Yasui Y, Hammond S, Bhatia S, Neglia JP, Stovall M, Srivastava D, Robison LL (2011) Occurrence of multiple subsequent neoplasms in long-term survivors of childhood cancer: a report from the childhood cancer survivor study. J Clin Oncol 29:3056–3064. doi:10.1200/JCO.2011.34.6585
82. Travis LB, Fossa SD, Schonfeld SJ, McMaster ML, Lynch CF, Storm H, Hall P, Holowaty E, Andersen A, Pukkala E, Andersson M, Kaijser M, Gospodarowicz M, Joensuu T, Cohen RJ, Boice JD Jr, Dores GM, Gilbert ES (2005) Second cancers among 40,576 testicular cancer patients: focus on long-term survivors. J Natl Cancer Inst 97:1354–1365. doi:10.1093/jnci/dji278
83. De Koning P, Neijt JP, Jennekens FG, Gispen WH (1987) Evaluation of cis-diamminedichloroplatinum (II) (cisplatin) neurotoxicity in rats. Toxicol Appl Pharmacol 89:81–87. doi:10.1016/0041-008X(87)90178-5
84. Marullo R, Werner E, Degtyareva N, Moore B, Altavilla G, Ramalingam SS, Doetsch PW (2013) Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. PLoS One 8:e81162. doi:10.1371/journal.pone.0081162
85. Luo J, Solimini NL, Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 136:823–837. doi:10.1016/j.cell.2009.02.024
86. Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF,

Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR (2007) Patterns of somatic mutation in human cancer genomes. Nature 446:153–158. doi:10.1038/nature05610
87. Capdeville R, Buchdunger E, Zimmermann J, Matter A (2002) Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat Rev Drug Discov 1:493–502. doi:10.1038/nrd839
88. Ashwell S, Zabludoff S (2008) DNA damage detection and repair pathways–recent advances with inhibitors of checkpoint kinases in cancer therapy. Clin Cancer Res 14:4032–4037. doi:10.1158/1078-0432.CCR-07-5138
89. Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer. Cancer Cell 12:9–22. doi:10.1016/j.ccr.2007.05.008
90. Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol 11:515–528. doi:10.1038/nrm2918
91. Stadtman ER (2001) Protein oxidation in aging and age-related diseases. Ann N Y Acad Sci 928:22–38. doi:10.1111/j.1749-6632.2001.tb05632.x
92. Soga S, Akinaga S, Shiotsu Y (2013) Hsp90 inhibitors as anti-cancer agents, from basic discoveries to clinical development. Curr Pharm Des 19:366–376. doi:10.2174/1381612811306030366
93. Shen M, Ping Dou Q (2013) Proteasome inhibition as a novel strategy for cancer treatment. In: Johnson DE (ed) Cell death signaling in cancer biology and treatment. Springer Sciences, New York, NY, pp 303–329
94. Groll M, Berkers CR, Ploegh HL, Ovaa H (2006) Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome. Structure 14:451–456. doi:10.1016/j.str.2005.11.019
95. D'Arcy P, Brnjic S, Olofsson MH, Fryknas M, Lindsten K, De Cesare M, Perego P, Sadeghi B, Hassan M, Larsson R, Linder S (2011) Inhibition of proteasome deubiquitinating activity as a new cancer therapy. Nat Med 17:1636–1640. doi:10.1038/nm.2536
96. Lee BH, Lee MJ, Park S, Oh DC, Elsasser S, Chen PC, Gartner C, Dimova N, Hanna J, Gygi SP, Wilson SM, King RW, Finley D (2010) Enhancement of proteasome activity by a small-molecule inhibitor of USP14. Nature 467:179–184. doi:10.1038/nature09299
97. Ceccarelli DF, Tang X, Pelletier B, Orlicky S, Xie W, Plantevin V, Neculai D, Chou YC, Ogunjimi A, Al-Hakim A, Varelas X, Koszela J, Wasney GA, Vedadi M, Dhe-Paganon S, Cox S, Xu S, Lopez-Girona A, Mercurio F, Wrana J, Durocher D, Meloche S, Webb DR, Tyers M, Sicheri F (2011) An allosteric inhibitor of the human Cdc34 ubiquitin-conjugating enzyme. Cell 145:1075–1087. doi:10.1016/j.cell.2011.05.039
98. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, Cullis CA, Doucette A, Garnsey JJ, Gaulin JL, Gershman RE, Lublinsky AR, McDonald A, Mizutani H, Narayanan U, Olhava EJ, Peluso S, Rezaei M, Sintchak MD, Talreja T, Thomas MP, Traore T, Vyskocil S, Weatherhead GS, Yu J, Zhang J, Dick LR, Claiborne CF, Rolfe M, Bolen JB, Langston SP (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 458:732–736. doi:10.1038/nature07884
99. Deshpande AJ, Bradner J, Armstrong SA (2012) Chromatin modifications as therapeutic targets in MLL-rearranged leukemia. Trends Immunol 33:563–570. doi:10.1016/j.it.2012.06.002
100. Chi P, Allis CD, Wang GG (2010) Covalent histone modifications–miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer 10:457–469. doi:10.1038/nrc2876
101. Kubota Y (2012) Tumor angiogenesis and anti-angiogenic therapy. Keio J Med 61:47–56. doi:10.2302/kjm.61.47
102. Risau W (1997) Mechanisms of angiogenesis. Nature 386:671–674. doi:10.1038/386671a0
103. Bikfalvi A, Bicknell R (2002) Recent advances in angiogenesis, anti-angiogenesis and vascular targeting. Trends Pharmacol Sci 23:576–582. doi:10.1016/S0165-6147(02)02109-0
104. Chung AS, Ferrara N (2011) Developmental and pathological angiogenesis. Annu Rev Cell Dev Biol 27:563–584. doi:10.1146/annurev-cellbio-092910-154002

105. Dayan F, Mazure NM, Brahimi-Horn MC, Pouyssegur J (2008) A dialogue between the hypoxia-inducible factor and the tumor microenvironment. Cancer Microenviron 1:53–68. doi:10.1007/s12307-008-0006-3
106. Nagy JA, Dvorak AM, Dvorak HF (2007) VEGF-A and the induction of pathological angiogenesis. Annu Rev Pathol 2:251–275. doi:10.1146/annurev.pathol.2.010506.134925
107. Hendrix MJ, Seftor EA, Hess AR, Seftor RE (2003) Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 3:411–421. doi:10.1038/nrc1092
108. Adams RH, Alitalo K (2007) Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 8:464–478. doi:10.1038/nrm2183
109. Shojaei F (2012) Anti-angiogenesis therapy in cancer: current challenges and future perspectives. Cancer Lett 320:130–137. doi:10.1016/j.canlet.2012.03.008
110. Deng T, Zhang L, Liu XJ, Xu JM, Bai YX, Wang Y, Han Y, Li YH, Ba Y (2013) Bevacizumab plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as the second-line therapy for patients with metastatic colorectal cancer, a multicenter study. Med Oncol 30:752. doi:10.1007/s12032-013-0752-z
111. Van Cutsem E, Tabernero J, Lakomy R, Prenen H, Prausova J, Macarulla T, Ruff P, van Hazel GA, Moiseyenko V, Ferry D, McKendrick J, Polikoff J, Tellier A, Castan R, Allegra C (2012) Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. J Clin Oncol 30:3499–3506. doi:10.1200/JCO.2012.42.8201
112. Smith NR, Baker D, Farren M, Pommier A, Swann R, Wang X, Mistry S, McDaid K, Kendrew J, Womack C, Wedge SR, Barry ST (2013) Tumor stromal architecture can define the intrinsic tumor response to VEGF-targeted therapy. Clin Cancer Res 19:6943–6956. doi:10.1158/1078-0432.CCR-13-1637
113. Vasudev NS, Reynolds AR (2014) Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. Angiogenesis. doi:10.1007/s10456-014-9420-y
114. Henkin J, Volpert OV (2011) Therapies using anti-angiogenic peptide mimetics of thrombospondin-1. Expert Opin Ther Targets 15:1369–1386. doi:10.1517/14728222.2011.640319
115. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60:1878–1886, PMID: 10766175
116. Damber JE, Vallbo C, Albertsson P, Lennernas B, Norrby K (2006) The anti-tumour effect of low-dose continuous chemotherapy may partly be mediated by thrombospondin. Cancer Chemother Pharmacol 58:354–360. doi:10.1007/s00280-005-0163-8
117. Yap R, Veliceasa D, Emmenegger U, Kerbel RS, McKay LM, Henkin J, Volpert OV (2005) Metronomic low-dose chemotherapy boosts CD95-dependent antiangiogenic effect of the thrombospondin peptide ABT-510: a complementation antiangiogenic strategy. Clin Cancer Res 11:6678–6685. doi:10.1158/1078-0432.CCR-05-0621
118. Streit M, Stephen AE, Hawighorst T, Matsuda K, Lange-Asschenfeldt B, Brown LF, Vacanti JP, Detmar M (2002) Systemic inhibition of tumor growth and angiogenesis by thrombospondin-2 using cell-based antiangiogenic gene therapy. Cancer Res 62:2004–2012, PMID: 11929817
119. Butler GS, Overall CM (2009) Proteomic identification of multitasking proteins in unexpected locations complicates drug targeting. Nat Rev Drug Discov 8:935–948. doi:10.1038/nrd2945
120. Hadler-Olsen E, Winberg JO, Uhlin-Hansen L (2013) Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets. Tumour Biol 34:2041–2051. doi:10.1007/s13277-013-0842-8
121. Cooney MM, van Heeckeren W, Bhakta S, Ortiz J, Remick SC (2006) Drug insight: vascular disrupting agents and angiogenesis–novel approaches for drug delivery. Nat Clin Pract Oncol 3:682–692. doi:10.1038/ncponc0663
122. Dark GG, Hill SA, Prise VE, Tozer GM, Pettit GR, Chaplin DJ (1997) Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. Cancer Res 57:1829–1834, PMID: 9157969

123. Zhou S, Kestell P, Baguley BC, Paxton JW (2002) 5,6-dimethylxanthenone-4-acetic acid (DMXAA): a new biological response modifier for cancer therapy. Invest New Drugs 20:281–295. doi:10.1023/A:1016215015530
124. Gajewski TF, Schreiber H, Fu YX (2013) Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 14:1014–1022. doi:10.1038/ni.2703
125. Engels B, Engelhard VH, Sidney J, Sette A, Binder DC, Liu RB, Kranz DM, Meredith SC, Rowley DA, Schreiber H (2013) Relapse or eradication of cancer is predicted by peptide-major histocompatibility complex affinity. Cancer Cell 23:516–526. doi:10.1016/j.ccr.2013.03.018
126. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med 19:747–752. doi:10.1038/nm.3161
127. Goldenberg DM (1993) Monoclonal antibodies in cancer detection and therapy. Am J Med 94:297–312. doi:10.1016/0002-9343(93)90062-T
128. Carter P (2001) Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 1:118–129. doi:10.1038/35101072
129. Monoclonal antibody therapy (2014). http://en.wikipedia.org/wiki/Monoclonal_antibody_therapy. Accessed Mar 2014.
130. Sharkey RM, Goldenberg DM (2011) Cancer radioimmunotherapy. Immunotherapy 3:349–370. doi:10.2217/imt.10.114
131. Pressman D, Korngold L (1953) The in vivo localization of anti-Wagner-osteogenic-sarcoma antibodies. Cancer 6:619–623, PMID: 13042784
132. DeNardo SJ, DeNardo GL, O'Grady LF, Macey DJ, Mills SL, Epstein AL, Peng JS, McGahan JP (1987) Treatment of a patient with B cell lymphoma by I-131 LYM-1 monoclonal antibodies. Int J Biol Markers 2:49–53, PMID: 3501448
133. Press OW, Eary JF, Badger CC, Martin PJ, Appelbaum FR, Levy R, Miller R, Brown S, Nelp WB, Krohn KA et al (1989) Treatment of refractory non-Hodgkin's lymphoma with radiolabeled MB-1 (anti-CD37) antibody. J Clin Oncol 7:1027–1038, PMID: 2666588
134. Kaminski MS, Zasadny KR, Francis IR, Milik AW, Ross CW, Moon SD, Crawford SM, Burgess JM, Petry NA, Butchko GM et al (1993) Radioimmunotherapy of B-cell lymphoma with [131I]anti-B1 (anti-CD20) antibody. N Engl J Med 329:459–465. doi:10.1056/NEJM199308123290703
135. Goldenberg DM (2002) Targeted therapy of cancer with radiolabeled antibodies. J Nucl Med 43:693–713, PMID: 11994535
136. Vacchelli E, Vitale I, Tartour E, Eggermont A, Sautes-Fridman C, Galon J, Zitvogel L, Kroemer G, Galluzzi L (2013) Trial watch: anticancer radioimmunotherapy. Oncoimmunology 2:e25595. doi:10.4161/onci.25595
137. www.clinicaltrials.gov. Accessed in Mar 2014
138. Almqvist Y, Orlova A, Sjöström A, Jensen HJ, Lundqvist H, Sundin A, Tolmachev V (2005) In vitro characterization of 211At-labeled antibody A33-a potential therapeutic agent against metastatic colorectal carcinoma. Cancer Biother Radiopharm 20:514–523. doi:10.1089/cbr.2005.20.514
139. Pickhard A, Piontek G, Seidl C, Kopping S, Blechert B, Misslbeck M, Brockhoff G, Bruchertseifer F, Morgenstern A, Essler M (2014) (2)(1)(3)Bi-anti-EGFR radioimmunoconjugates and X-ray irradiation trigger different cell death pathways in squamous cell carcinoma cells. Nucl Med Biol 41:68–76. doi:10.1016/j.nucmedbio.2013.09.010
140. Qu C, Li Y, Song Y, Rizvi S, Raja C, Zhang D, Samra J, Smith R, Perkins A, Apostolidis C (2004) MUC1 expression in primary and metastatic pancreatic cancer cells for in vitro treatment by 213Bi-C595 radioimmunoconjugate. Br J Cancer 91:2086–2093. doi:10.1038/sj.bjc.6602232
141. Zhang DY, Li Y, Rizvi SMA, Qu C, Kearsley J, Allen BJ (2005) Cytotoxicity of breast cancer cells overexpressing HER2/neu by ^{213}Bi-Herceptin radioimmunoconjugate. Cancer Lett 218:181–190. doi:10.1016/j.canlet.2004.07.050

142. Li Y, Song E, Abbas Rizvi SM, Power CA, Beretov J, Raja C, Cozzi PJ, Morgenstern A, Apostolidis C, Allen BJ, Russell PJ (2009) Inhibition of micrometastatic prostate cancer cell spread in animal models by 213Bilabeled multiple targeted alpha radioimmunoconjugates. Clin Cancer Res 15:865–875. doi:10.1158/1078-0432.CCR-08-1203
143. Pavlinkova G, Booth BJ, Batra SK, Colcher D (1999) Radioimmunotherapy of human colon cancer xenografts using a dimeric single-chain Fv antibody construct. Clin Cancer Res 5:2613–2619, PMID: 10499640
144. Hu M, Chen P, Wang J, Scollard DA, Vallis KA, Reilly RM (2007) 123I-labeled HIV-1 tat peptide radioimmunoconjugates are imported into the nucleus of human breast cancer cells and functionally interact in vitro and in vivo with the cyclin-dependent kinase inhibitor, p21WAF-1/Cip-1. Eur J Nucl Med Mol Imaging 34:368–377. doi:10.1007/s00259-006-0189-0
145. Brouwers AH, van Eerd JE, Frielink C, Oosterwijk E, Oyen WJ, Corstens FH, Boerman OC (2004) Optimization of radioimmunotherapy of renal cell carcinoma: labeling of monoclonal antibody cG250 with 131I, 90Y, 177Lu, or 186Re. J Nucl Med 45:327–337, PMID: 14960657
146. Sharkey RM, Blumenthal RD, Behr TM, Wong GY, Haywood L, Forman D, Griffiths GL, Goldenberg DM (1997) Selection of radioimmunoconjugates for the therapy of well-established or micrometastatic colon carcinoma. Int J Cancer 72:477–485. doi:10.1002/(SICI)1097-0215(19970729)72:3<477::AID-IJC16>3.0.CO;2-9
147. Cornelissen B, Darbar S, Kersemans V, Allen D, Falzone N, Barbeau J, Smart S, Vallis KA (2012) Amplification of DNA damage by a gammaH2AX-targeted radiopharmaceutical. Nucl Med Biol 39:1142–1151. doi:10.1016/j.nucmedbio.2012.06.001
148. Chinn PC, Morena RA, Santoro DA, Kazules T, Kashmiri SV, Schlom J, Hanna N, Braslawsky G (2006) Pharmacokinetics and tumor localization of 111In-labeled HuCC49ΔCH2 in BALB/c mice and athymic murine colon carcinoma xenograft. Cancer Biother Radiopharm 21:106–116. doi:10.1089/cbr.2006.21.106
149. Cornelissen B, McLarty K, Kersemans V, Scollard DA, Reilly RM (2008) Properties of [(111)In]-labeled HIV-1 tat peptide radioimmunoconjugates in tumor-bearing mice following intravenous or intratumoral injection. Nucl Med Biol 35:101–110. doi:10.1016/j.nucmedbio.2007.09.007
150. Liu P, Boyle AJ, Lu Y, Reilly RM, Winnik MA (2012) Biotinylated polyacrylamide-based metal-chelating polymers and their influence on antigen recognition following conjugation to a trastuzumab Fab fragment. Biomacromolecules 13:2831–2842. doi:10.1021/bm300843u
151. Sandstrom K, Nestor M, Ekberg T, Engstrom M, Anniko M, Lundqvist H (2008) Targeting CD44v6 expressed in head and neck squamous cell carcinoma: preclinical characterization of an 111In-labeled monoclonal antibody. Tumour Biol 29:137–144. doi:10.1159/000143399
152. Price EW, Zeglis BM, Cawthray JF, Ramogida CF, Ramos N, Lewis JS, Adam MJ, Orvig C (2013) H(4)octapa-trastuzumab: versatile acyclic chelate system for 111In and 177Lu imaging and therapy. J Am Chem Soc 135:12707–12721. doi:10.1021/ja4049493
153. Tempero M, Leichner P, Baranowska-Kortylewicz J, Harrison K, Augustine S, Schlom J, Anderson J, Wisecarver J, Colcher D (2000) High-dose therapy with 90Yttrium-labeled monoclonal antibody CC49: a phase I trial. Clin Cancer Res 6:3095–3102, PMID: 10955789
154. Fischer E, Grunberg J, Cohrs S, Hohn A, Waldner-Knogler K, Jeger S, Zimmermann K, Novak-Hofer I, Schibli R (2012) L1-CAM-targeted antibody therapy and (177) Lu-radioimmunotherapy of disseminated ovarian cancer. Int J Cancer 130:2715–2721. doi:10.1002/ijc.26321
155. Nilsson R, Eriksson SE, Sjogren HO, Tennvall J (2011) Different toxicity profiles for drug- versus radionuclide-conjugated BR96 monoclonal antibodies in a syngeneic rat colon carcinoma model. Acta Oncol 50:711–718. doi:10.3109/0284186X.2010.547215
156. Schneider N, Lobaugh M, Tan Z, Sandwall P, Chen P, Glover S, Cui L, Murry M, Dong Z, Torgue J (2013) Biodistribution of 212Pb conjugated trastuzumab in mice. J Radioanal Nucl Chem 296:75–81. doi:10.1007/s10967-012-2243-7
157. Mohsin H, Jia F, Sivaguru G, Hudson MJ, Shelton TD, Hoffman TJ, Cutler CS, Ketring AR, Athey PS, Simón J (2006) Radiolanthanide-labeled monoclonal antibody CC49 for

radioimmunotherapy of cancer: biological comparison of DOTA conjugates and 149Pm, 166Ho, and 177Lu. Bioconjug Chem 17:485–492. doi:10.1021/bc0502356
158. Stroomer JW, Roos JC, Sproll M, Quak JJ, Heider KH, Wilhelm BJ, Castelijns JA, Meyer R, Kwakkelstein MO, Snow GB, Adolf GR, van Dongen GA (2000) Safety and biodistribution of 99mTechnetium-labeled anti-CD44v6 monoclonal antibody BIWA 1 in head and neck cancer patients. Clin Cancer Res 6:3046–3055, PMID: 10955783
159. Dahle J, Borrebaek J, Jonasdottir TJ, Hjelmerud AK, Melhus KB, Bruland OS, Press OW, Larsen RH (2007) Targeted cancer therapy with a novel low-dose rate alpha-emitting radioimmunoconjugate. Blood 110:2049–2056. doi:10.1182/blood-2007-01-066803
160. Heyerdahl H, Abbas N, Brevik EM, Mollatt C, Dahle J (2012) Fractionated therapy of HER2-expressing breast and ovarian cancer xenografts in mice with targeted alpha emitting 227Th-DOTA-p-benzyl-trastuzumab. PLoS One 7:e42345. doi:10.1371/journal.pone.0042345
161. Maraveyas A, Snook D, Hird V, Kosmas C, Meares CF, Lambert HE, Epenetos AA (1994) Pharmacokinetics and toxicity of an yttrium-90-CITC-DTPA-HMFG1 radioimmunoconjugate for intraperitoneal radioimmunotherapy of ovarian cancer. Cancer 73:1067–1075, PMID: 8306249
162. Jia F, Liu X, Li L, Mallapragada S, Narasimhan B, Wang Q (2013) Multifunctional nanoparticles for targeted delivery of immune activating and cancer therapeutic agents. J Control Release 172:1020–1034. doi:10.1016/j.jconrel.2013.10.012
163. Kitson SL, Cuccurullo V, Moody TS, Mansi L (2013) Radionuclide antibody-conjugates, a targeted therapy towards cancer. Curr Radiopharm 6:57–71. doi:10.2174/1874471011306020001
164. Zhang L, Chen H, Wang L, Liu T, Yeh J, Lu G, Yang L, Mao H (2010) Delivery of therapeutic radioisotopes using nanoparticle platforms: potential benefit in systemic radiation therapy. Nanotechnol Sci Appl 3:159–170. doi:10.2147/NSA.S7462
165. Barrett T, Ravizzini G, Choyke PL, Kobayashi H (2009) Dendrimers in medical nanotechnology. IEEE Eng Med Biol Mag 28:12–22. doi:10.1109/MEMB.2008.931012
166. Veiseh O, Kievit FM, Ellenbogen RG, Zhang M (2011) Cancer cell invasion: treatment and monitoring opportunities in nanomedicine. Adv Drug Deliv Rev 63:582–596. doi:10.1016/j.addr.2011.01.010
167. Li L, Wartchow CA, Danthi SN, Shen Z, Dechene N, Pease J, Choi HS, Doede T, Chu P, Ning S, Lee DY, Bednarski MD, Knox SJ (2004) A novel antiangiogenesis therapy using an integrin antagonist or anti-Flk-1 antibody coated 90Y-labeled nanoparticles. Int J Radiat Oncol Biol Phys 58:1215–1227. doi:10.1016/j.ijrobp.2003.10.057
168. Belhaj-Tayeb H, Briane D, Vergote J, Kothan S, Leger G, Bendada SE, Tofighi M, Tamgac F, Cao A, Moretti JL (2003) In vitro and in vivo study of 99mTc-MIBI encapsulated in PEG-liposomes: a promising radiotracer for tumour imaging. Eur J Nucl Med Mol Imaging 30:502–509. doi:10.1007/s00259-002-1038-4
169. Kobayashi H, Wu C, Kim MK, Paik CH, Carrasquillo JA, Brechbiel MW (1999) Evaluation of the in vivo biodistribution of indium-111 and yttrium-88 labeled dendrimer-1B4M-DTPA and its conjugation with anti-Tac monoclonal antibody. Bioconjug Chem 10:103–111. doi:10.1021/bc980091d
170. Torchilin VP (2002) PEG based micelles as carriers of contrast agents for different imaging modalities. Adv Drug Deliv Rev 54:235–252. doi:10.1016/S0169-409X(02)00019-4
171. Hallahan D, Geng L, Qu S, Scarfone C, Giorgio T, Donnelly E, Gao X, Clanton J (2003) Integrin-mediated targeting of drug delivery to irradiated tumor blood vessels. Cancer Cell 3:63–74. doi:10.1016/S1535-6108(02)00238-6
172. Hu G, Lijowski M, Zhang H, Partlow KC, Caruthers SD, Kiefer G, Gulyas G, Athey P, Scott MJ, Wickline SA, Lanza GM (2007) Imaging of Vx-2 rabbit tumors with alpha(nu)beta3-integrin-targeted 111In nanoparticles. Int J Cancer 120:1951–1957. doi:10.1002/ijc.22581
173. Chang YJ, Chang CH, Yu CY, Chang TJ, Chen LC, Chen MH, Lee TW, Ting G (2010) Therapeutic efficacy and microSPECT/CT imaging of 188Re-DXR-liposome in a C26 murine colon carcinoma solid tumor model. Nucl Med Biol 37:95–104. doi:10.1016/j.nucmedbio.2009.08.006

174. Vicente S, Goins BA, Sanchez A, Alonso MJ, Phillips WT (2014) Biodistribution and lymph node retention of polysaccharide-based immunostimulating nanocapsules. Vaccine 32:1685–1692. doi:10.1016/j.vaccine.2014.01.059
175. Chen J, Wu H, Han D, Xie C (2006) Using anti-VEGF McAb and magnetic nanoparticles as double-targeting vector for the radioimmunotherapy of liver cancer. Cancer Lett 231:169–175. doi:10.1016/j.canlet.2005.01.024
176. Wu H, Wang J, Wang Z, Fisher DR, Lin Y (2008) Apoferritin-templated yttrium phosphate nanoparticle conjugates for radioimmunotherapy of cancers. J Nanosci Nanotechnol 8:2316–2322, PMID: 18572643
177. Woodward JD, Kennel SJ, Mirzadeh S, Dai S, Wall JS, Richey T, Avenell J, Rondinone AJ (2007) In vivo SPECT/CT imaging and biodistribution using radioactive Cd125mTe/ZnS nanoparticles. Nanotechnology 18:175103. doi:10.1088/0957-4484/18/17/175103
178. Liu Z, Cai W, He L, Nakayama N, Chen K, Sun X, Chen X, Dai H (2007) In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. Nat Nanotechnol 2:47–52. doi:10.1038/nnano.2006.170
179. Bandekar A, Zhu C, Jindal R, Bruchertseifer F, Morgenstern A, Sofou S (2014) Anti-prostate-specific membrane antigen liposomes loaded with 225Ac for potential targeted antivascular alpha-particle therapy of cancer. J Nucl Med 55:107–114. doi:10.2967/jnumed.113.125476
180. Wayne AS, Fitzgerald DJ, Kreitman RJ, Pastan I (2014) Immunotoxins for leukemia. Blood. doi:10.1182/blood-2014-01-492256
181. Antignani A, Fitzgerald D (2013) Immunotoxins: the role of the toxin. Toxins (Basel) 5:1486–1502. doi:10.3390/toxins5081486
182. Olsen E, Duvic M, Frankel A, Kim Y, Martin A, Vonderheid E, Jegasothy B, Wood G, Gordon M, Heald P, Oseroff A, Pinter-Brown L, Bowen G, Kuzel T, Fivenson D, Foss F, Glode M, Molina A, Knobler E, Stewart S, Cooper K, Stevens S, Craig F, Reuben J, Bacha P, Nichols J (2001) Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. J Clin Oncol 19:376–388, PMID: 11208829
183. Jansen FK, Bourrie B, Casellas P, Dussossoy D, Gros O, Vic P, Vidal H, Gros P (1988) Toxin selection and modification: utilization of the A chain of ricin. Cancer Treat Res 37:97–111. doi:10.1007/978-1-4613-1083-9_7
184. Hudson TH, Neville DM Jr (1988) Enhancement of immunotoxin action: manipulation of the cellular routing of proteins. Cancer Treat Res 37:371–389. doi:10.1007/978-1-4613-1083-9_20
185. Casellas P, Jansen FK (1988) Immunotoxin enhancers. Cancer Treat Res 37:351–369. doi:10.1007/978-1-4613-1083-9_19
186. Kantarjian H, Thomas D, Wayne AS, O'Brien S (2012) Monoclonal antibody-based therapies: a new dawn in the treatment of acute lymphoblastic leukemia. J Clin Oncol 30:3876–3883. doi:10.1200/JCO.2012.41.6768
187. Messmann RA, Vitetta ES, Headlee D, Senderowicz AM, Figg WD, Schindler J, Michiel DF, Creekmore S, Steinberg SM, Kohler D, Jaffe ES, Stetler-Stevenson M, Chen H, Ghetie V, Sausville EA (2000) A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylated ricin A chain (dgA) and IgG-RFB4-dgA (Combotox) in patients with refractory CD19(+), CD22(+) B cell lymphoma. Clin Cancer Res 6:1302–1313, PMID: 10778955
188. Grossbard ML, Multani PS, Freedman AS, O'Day S, Gribben JG, Rhuda C, Neuberg D, Nadler LM (1999) A Phase II study of adjuvant therapy with anti-B4-blocked ricin after autologous bone marrow transplantation for patients with relapsed B-cell non-Hodgkin's lymphoma. Clin Cancer Res 5:2392–2398, PMID: 10499609
189. Vitetta ES (2000) Immunotoxins and vascular leak syndrome. Cancer J 6(Suppl 3):S218–224, PMID: 10874491
190. Siegall CB, Liggitt D, Chace D, Mixan B, Sugai J, Davidson T, Steinitz M (1997) Characterization of vascular leak syndrome induced by the toxin component of Pseudomonas exotoxin-based immunotoxins and its potential inhibition with nonsteroidal anti-inflammatory drugs. Clin Cancer Res 3:339–345, PMID: 9815690

191. Hu RG, Zhai QW, He WJ, Mei L, Liu WY (2002) Bioactivities of ricin retained and its immunoreactivity to anti-ricin polyclonal antibodies alleviated through pegylation. Int J Biochem Cell Biol 34:396–402. doi:10.1016/S1357-2725(01)00128-5
192. Oraki Kohshour M, Mirzaie S, Zeinali M, Amin M, Said Hakhamaneshi M, Jalaili A, Mosaveri N, Jamalan M (2014) Ablation of breast cancer cells using trastuzumab-functionalized multi-walled carbon nanotubes and trastuzumab-diphtheria toxin conjugate. Chem Biol Drug Des 83:259–265. doi:10.1111/cbdd.12244
193. Ramakrishnan S, Olson TA, Bautch VL, Mohanraj D (1996) Vascular endothelial growth factor-toxin conjugate specifically inhibits KDR/flk-1-positive endothelial cell proliferation in vitro and angiogenesis in vivo. Cancer Res 56:1324–1330, PMID: 8640821
194. Selbo PK, Kaalhus O, Sivam G, Berg K (2001) 5-Aminolevulinic acid-based photochemical internalization of the immunotoxin MOC31-gelonin generates synergistic cytotoxic effects in vitro. Photochem Photobiol 74:303–310. doi:10.1562/0031-8655(2001)0740303AABPIO2.0.CO2
195. Schiffer S, Letzian S, Jost E, Mladenov R, Hristodorov D, Huhn M, Fischer R, Barth S, Thepen T (2013) Granzyme M as a novel effector molecule for human cytolytic fusion proteins: CD64-specific cytotoxicity of Gm-H22 (scFv) against leukemic cells. Cancer Lett 341:178–185. doi:10.1016/j.canlet.2013.08.005
196. Bergelt S, Frost S, Lilie H (2009) Listeriolysin O as cytotoxic component of an immunotoxin. Protein Sci 18:1210–1220. doi:10.1002/pro.130
197. Hassan R, Viner JL, Wang QC, Margulies I, Kreitman RJ, Pastan I (2000) Anti-tumor activity of K1-LysPE38QQR, an immunotoxin targeting mesothelin, a cell-surface antigen overexpressed in ovarian cancer and malignant mesothelioma. J Immunother 23:473–479. doi:10.1097/00002371-200007000-00011
198. Bruell D, Stocker M, Huhn M, Redding N, Kupper M, Schumacher P, Paetz A, Bruns CJ, Haisma HJ, Fischer R, Finnern R, Barth S (2003) The recombinant anti-EGF receptor immunotoxin 425(scFv)-ETA′ suppresses growth of a highly metastatic pancreatic carcinoma cell line. Int J Oncol 23:1179–1186, PMID: 12964002
199. Park JH, Kwon HW, Chung HK, Kim IH, Ahn K, Choi EJ, Pastan I, Choe M (2001) A divalent recombinant immunotoxin formed by a disulfide bond between the extension peptide chains. Mol Cells 12:398–402, PMID: 11804341
200. Beers R, Chowdhury P, Bigner D, Pastan I (2000) Immunotoxins with increased activity against epidermal growth factor receptor vIII-expressing cells produced by antibody phage display. Clin Cancer Res 6:2835–2843, PMID: 10914732
201. Salvatore G, Beers R, Margulies I, Kreitman RJ, Pastan I (2002) Improved cytotoxic activity toward cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. Clin Cancer Res 8:995–1002, PMID: 11948105
202. Akamatsu Y, Murphy JC, Nolan KF, Thomas P, Kreitman RJ, Leung SO, Junghans RP (1998) A single-chain immunotoxin against carcinoembryonic antigen that suppresses growth of colorectal carcinoma cells. Clin Cancer Res 4:2825–2832, PMID: 9829749
203. Liu W, Onda M, Kim C, Xiang L, Weldon JE, Lee B, Pastan I (2012) A recombinant immunotoxin engineered for increased stability by adding a disulfide bond has decreased immunogenicity. Protein Eng Des Sel 25:1–6. doi:10.1093/protein/gzr053
204. Wang L, Liu B, Schmidt M, Lu Y, Wels W, Fan Z (2001) Antitumor effect of an HER2-specific antibody-toxin fusion protein on human prostate cancer cells. Prostate 47:21–28. doi:10.1002/pros.1043
205. Gadadhar S, Karande AA (2013) Abrin immunotoxin: targeted cytotoxicity and intracellular trafficking pathway. PLoS One 8:e58304. doi:10.1371/journal.pone.0058304
206. Martinez-Torrecuadrada JL, Cheung LH, Lopez-Serra P, Barderas R, Canamero M, Ferreiro S, Rosenblum MG, Casal JI (2008) Antitumor activity of fibroblast growth factor receptor 3-specific immunotoxins in a xenograft mouse model of bladder carcinoma is mediated by apoptosis. Mol Cancer Ther 7:862–873. doi:10.1158/1535-7163.MCT-07-0394
207. Fracasso G, Bellisola G, Cingarlini S, Castelletti D, Prayer-Galetti T, Pagano F, Tridente G, Colombatti M (2002) Anti-tumor effects of toxins targeted to the prostate specific membrane antigen. Prostate 53:9–23. doi:10.1002/pros.10117

208. Gilabert-Oriol R, Thakur M, von Mallinckrodt B, Hug T, Wiesner B, Eichhorst J, Melzig MF, Fuchs H, Weng A (2013) Modified trastuzumab and cetuximab mediate efficient toxin delivery while retaining antibody-dependent cell-mediated cytotoxicity in target cells. Mol Pharm 10:4347–4357. doi:10.1021/mp400444q
209. Wei BR, Ghetie MA, Vitetta ES (2000) The combined use of an immunotoxin and a radioimmunoconjugate to treat disseminated human B-cell lymphoma in immunodeficient mice. Clin Cancer Res 6:631–642, PMID: 10690549
210. Gillies SD, Young D, Lo KM, Roberts S (1993) Biological activity and in vivo clearance of antitumor antibody/cytokine fusion proteins. Bioconjug Chem 4:230–235. doi:10.1021/bc00021a008
211. Schrama D, Reisfeld RA, Becker JC (2006) Antibody targeted drugs as cancer therapeutics. Nat Rev Drug Discov 5:147–159. doi:10.1038/nrd1957
212. Manusama ER, Nooijen PT, Ten Hagen TL, Van Der Veen AH, De Vries MW, De Wilt JH, Van Ijken MG, Marquet RL, Eggermont AM (1998) Tumor necrosis factor-alpha in isolated perfusion systems in the treatment of cancer: the Rotterdam preclinical-clinical program. Semin Surg Oncol 14:232–237. doi:10.1002/(SICI)1098-2388(199804/05)14:3<232::AID-SSU7>3.0.CO;2-9
213. Cohen J (1995) IL-12 deaths: explanation and a puzzle. Science 270:908. doi:10.1126/science.270.5238.908a
214. Mattijssen V, De Mulder PH, De Graeff A, Hupperets PS, Joosten F, Ruiter DJ, Bier H, Palmer PA, Van den Broek P (1994) Intratumoral PEG-interleukin-2 therapy in patients with locoregionally recurrent head and neck squamous-cell carcinoma. Ann Oncol 5:957–960, PMID 7696170
215. Lienard D, Ewalenko P, Delmotte JJ, Renard N, Lejeune FJ (1992) High-dose recombinant tumor necrosis factor alpha in combination with interferon gamma and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. J Clin Oncol 10:52–60, PMID: 1727926
216. Atkins MB, Kunkel L, Sznol M, Rosenberg SA (2000) High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update. Cancer J Sci Am 6(Suppl 1):S11–S14, PMID: 10685652
217. Becker JC, Pancook JD, Gillies SD, Furukawa K, Reisfeld RA (1996) T cell-mediated eradication of murine metastatic melanoma induced by targeted interleukin 2 therapy. J Exp Med 183:2361–2366, PMID: 8642346
218. Halin C, Rondini S, Nilsson F, Berndt A, Kosmehl H, Zardi L, Neri D (2002) Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. Nat Biotechnol 20:264–269. doi:10.1038/nbt0302-264
219. List T, Neri D (2013) Immunocytokines: a review of molecules in clinical development for cancer therapy. Clin Pharmacol 5:29–45. doi:10.2147/CPAA.S49231
220. Schrama D, Xiang R, Eggert AO, Andersen MH, Pedersen Ls LO, Kampgen E, Schumacher TN, Reisfeld RR, Becker JC (2004) Shift from systemic to site-specific memory by tumor-targeted IL-2. J Immunol 172:5843–5850, PMID: 15128763
221. Schliemann C, Palumbo A, Zuberbuhler K, Villa A, Kaspar M, Trachsel E, Klapper W, Menssen HD, Neri D (2009) Complete eradication of human B-cell lymphoma xenografts using rituximab in combination with the immunocytokine L19-IL2. Blood 113:2275–2283. doi:10.1182/blood-2008-05-160747
222. Schwager K, Hemmerle T, Aebischer D, Neri D (2013) The immunocytokine L19-IL2 eradicates cancer when used in combination with CTLA-4 blockade or with L19-TNF. J Invest Dermatol 133:751–758. doi:10.1038/jid.2012.376
223. Bauer J, Namineni S, Reisinger F, Zoller J, Yuan D, Heikenwalder M (2012) Lymphotoxin, NF-kB, and cancer: the dark side of cytokines. Dig Dis 30:453–468. doi:10.1159/000341690
224. Huang X, Ye D, Thorpe PE (2011) Enhancing the potency of a whole-cell breast cancer vaccine in mice with an antibody-IL-2 immunocytokine that targets exposed phosphatidylserine. Vaccine 29:4785–4793. doi:10.1016/j.vaccine.2011.04.082
225. Ziebarth AJ, Felder MA, Harter J, Connor JP (2012) Uterine leiomyosarcoma diffusely express disialoganglioside GD2 and bind the therapeutic immunocytokine 14.18-IL2: implications for immunotherapy. Cancer Immunol Immunother 61:1149–1153

226. Moschetta M, Pretto F, Berndt A, Galler K, Richter P, Bassi A, Oliva P, Micotti E, Valbusa G, Schwager K, Kaspar M, Trachsel E, Kosmehl H, Bani MR, Neri D, Giavazzi R (2012) Paclitaxel enhances therapeutic efficacy of the F8-IL2 immunocytokine to EDA-fibronectin-positive metastatic human melanoma xenografts. Cancer Res 72:1814–1824. doi:10.1158/0008-5472.CAN-11-1919
227. Pedretti M, Verpelli C, Marlind J, Bertani G, Sala C, Neri D, Bello L (2010) Combination of temozolomide with immunocytokine F16-IL2 for the treatment of glioblastoma. Br J Cancer 103:827–836. doi:10.1038/sj.bjc.6605832
228. Frey K, Schliemann C, Schwager K, Giavazzi R, Johannsen M, Neri D (2010) The immunocytokine F8-IL2 improves the therapeutic performance of sunitinib in a mouse model of renal cell carcinoma. J Urol 184:2540–2548. doi:10.1016/j.juro.2010.07.030
229. Hank JA, Gan J, Ryu H, Ostendorf A, Stauder MC, Sternberg A, Albertini M, Lo KM, Gillies SD, Eickhoff J, Sondel PM (2009) Immunogenicity of the hu14.18-IL2 immunocytokine molecule in adults with melanoma and children with neuroblastoma. Clin Cancer Res 15:5923–5930
230. Holden SA, Lan Y, Pardo AM, Wesolowski JS, Gillies SD (2001) Augmentation of antitumor activity of an antibody-interleukin 2 immunocytokine with chemotherapeutic agents. Clin Cancer Res 7:2862–2869, PMID: 11555604
231. Johnson EE, Yamane BH, Buhtoiarov IN, Lum HD, Rakhmilevich AL, Mahvi DM, Gillies SD, Sondel PM (2009) Radiofrequency ablation combined with KS-IL2 immunocytokine (EMD 273066) results in an enhanced antitumor effect against murine colon adenocarcinoma. Clin Cancer Res 15:4875–4884. doi:10.1158/1078-0432.CCR-09-0110
232. Pasche N, Woytschak J, Wulhfard S, Villa A, Frey K, Neri D (2011) Cloning and characterization of novel tumor-targeting immunocytokines based on murine IL7. J Biotechnol 154:84–92. doi:10.1016/j.jbiotec.2011.04.003
233. Fallon J, Tighe R, Kradjian G, Guzman W, Bernhardt A, Neuteboom B, Lan Y, Sabzevari H, Schlom J, Greiner JW (2014) The immunocytokine NHS-IL12 as a potential cancer therapeutic. Oncotarget 5:1869–1884, PMID: 24681847
234. Kim H, Gao W, Ho M (2013) Novel immunocytokine IL12-SS1 (Fv) inhibits mesothelioma tumor growth in nude mice. PLoS One 8:e81919. doi:10.1371/journal.pone.0081919
235. Gillessen S, Gnad-Vogt US, Gallerani E, Beck J, Sessa C, Omlin A, Mattiacci MR, Liedert B, Kramer D, Laurent J, Speiser DE, Stupp R (2013) A phase I dose-escalation study of the immunocytokine EMD 521873 (Selectikine) in patients with advanced solid tumours. Eur J Cancer 49:35–44. doi:10.1016/j.ejca.2012.07.015
236. Kaspar M, Trachsel E, Neri D (2007) The antibody-mediated targeted delivery of interleukin-15 and GM-CSF to the tumor neovasculature inhibits tumor growth and metastasis. Cancer Res 67:4940–4948. doi:10.1158/0008-5472.CAN-07-0283
237. Liu D, Chang CH, Rossi EA, Cardillo TM, Goldenberg DM (2013) Interferon-lambda1 linked to a stabilized dimer of Fab potently enhances both antitumor and antiviral activities in targeted cells. PLoS One 8:e63940. doi:10.1371/journal.pone.0063940
238. Chen P, Nogusa S, Thapa RJ, Shaller C, Simmons H, Peri S, Adams GP, Balachandran S (2013) Anti-CD70 immunocytokines for exploitation of interferon-gamma-induced RIP1-dependent necrosis in renal cell carcinoma. PLoS One 8:e61446. doi:10.1371/journal.pone.0061446
239. Lyu MA, Kurzrock R, Rosenblum MG (2008) The immunocytokine scFv23/TNF targeting HER-2/neu induces synergistic cytotoxic effects with 5-fluorouracil in TNF-resistant pancreatic cancer cell lines. Biochem Pharmacol 75:836–846. doi:10.1016/j.bcp.2007.10.013
240. Bagshawe KD (2006) Antibody-directed enzyme prodrug therapy (ADEPT) for cancer. Expert Rev Anticancer Ther 6:1421–1431. doi:10.1586/14737140.6.10.1421
241. Cheng TL, Wei SL, Chen BM, Chern JW, Wu MF, Liu PW, Roffler SR (1999) Bystander killing of tumour cells by antibody-targeted enzymatic activation of a glucuronide prodrug. Br J Cancer 79:1378–1385. doi:10.1038/sj.bjc.6690221
242. Sharma SK (2013) Antibody-directed enzyme prodrug therapy (ADEPT). In: Schmidt SR (ed) Fusion proteins technologies for biopharmaceuticals. Wiley, New Jersey, pp 354–363

243. Begent RH, Bagshawe KD, Green AJ, Searle F (1987) The clinical value of imaging with antibody to human chorionic gonadotrophin in the detection of residual choriocarcinoma. Br J Cancer 55:657–660. doi:10.1038/bjc.1987.134
244. Bagshawe KD (2012) Antibody-directed enzyme prodrug therapy (ADEPT) – basic principles and its practice so far. In: Kratz F, Senter P, Steinhagen H (eds) Drug delivery in oncology. Wiley, Germany, pp 169–186
245. Baker M, Carr F (2010) Pre-clinical considerations in the assessment of immunogenicity for protein therapeutics. Curr Drug Saf 5:308–313. doi:10.2174/157488610792246000
246. Holgate RG, Baker MP (2009) Circumventing immunogenicity in the development of therapeutic antibodies. IDrugs 12:233–237, PMID: 19350467
247. Smith GK, Banks S, Blumenkopf TA, Cory M, Humphreys J, Laethem RM, Miller J, Moxham CP, Mullin R, Ray PH, Walton LM, Wolfe LA 3rd (1997) Toward antibody-directed enzyme prodrug therapy with the T268G mutant of human carboxypeptidase A1 and novel in vivo stable prodrugs of methotrexate. J Biol Chem 272:15804–15816, PMID: 9188478
248. Syrigos KN, Rowlinson-Busza G, Epenetos AA (1998) In vitro cytotoxicity following specific activation of amygdalin by beta-glucosidase conjugated to a bladder cancer-associated monoclonal antibody. Int J Cancer 78:712–719. doi:10.1002/(SICI)1097-0215(19981209)78:6<712::AID-IJC8>3.0.CO;2-D
249. Chen KC, Wu SY, Leu YL, Prijovich ZM, Chen BM, Wang HE, Cheng TL, Roffler SR (2011) A humanized immunoenzyme with enhanced activity for glucuronide prodrug activation in the tumor microenvironment. Bioconjug Chem 22:938–948. doi:10.1021/bc1005784
250. Alderson RF, Toki BE, Roberge M, Geng W, Basler J, Chin R, Liu A, Ueda R, Hodges D, Escandon E (2006) Characterization of a CC49-based single-chain fragment-β-lactamase fusion protein for antibody-directed enzyme prodrug therapy (ADEPT). Bioconjug Chem 17:410–418. doi:10.1021/bc0503521
251. Wang H, Shi PJ, Wu MF, Li N, Zhou XL, Fan FY (2010) Construction, expression and functional characterization of the beta-lactamase with alphav integrin ligands. Protein Pept Lett 17:1562–1565. doi:10.2174/0929866511009011562
252. Hao XK, Liu JY, Yue QH, Wu GJ, Bai YJ, Yin Y (2006) In vitro and in vivo prodrug therapy of prostate cancer using anti-gamma-Sm-scFv/hCPA fusion protein. Prostate 66:858–866. doi:10.1002/pros.20402
253. Deckert PM, Bornmann WG, Ritter G, Williams C Jr, Franke J, Keilholz U, Thiel E, Old LJ, Bertino JR, Welt S (2004) Specific tumour localisation of a huA33 antibody–carboxypeptidase A conjugate and activation of methotrexate-phenylalanine. Int J Oncol 24:1289–1295, PMID: 15067353
254. Sharma SK, Bagshawe KD, Burke PJ, Boden JA, Rogers GT, Springer CJ, Melton RG, Sherwood RF (1994) Galactosylated antibodies and antibody-enzyme conjugates in antibody-directed enzyme prodrug therapy. Cancer 73:1114–1120, PMID: 8306255
255. Webley SD, Francis RJ, Pedley RB, Sharma SK, Begent RH, Hartley JA, Hochhauser D (2001) Measurement of the critical DNA lesions produced by antibody-directed enzyme prodrug therapy (ADEPT) in vitro, in vivo and in clinical material. Br J Cancer 84:1671–1676. doi:10.1054/bjoc.2001.1843
256. Pedley RB, Sharma SK, Boxer GM, Boden R, Stribbling SM, Davies L, Springer CJ, Begent RH (1999) Enhancement of antibody-directed enzyme prodrug therapy in colorectal xenografts by an antivascular agent. Cancer Res 59:3998–4003, PMID: 10463598
257. Mayer A, Sharma SK, Tolner B, Minton NP, Purdy D, Amlot P, Tharakan G, Begent RH, Chester KA (2004) Modifying an immunogenic epitope on a therapeutic protein: a step towards an improved system for antibody-directed enzyme prodrug therapy (ADEPT). Br J Cancer 90:2402–2410. doi:10.1038/sj.bjc.6601888
258. Park JI, Cao L, Platt VM, Huang Z, Stull RA, Dy EE, Sperinde JJ, Yokoyama JS, Szoka FC (2009) Antitumor therapy mediated by 5-fluorocytosine and a recombinant fusion protein containing TSG-6 hyaluronan binding domain and yeast cytosine deaminase. Mol Pharm 6:801–812. doi:10.1021/mp800013c

259. Coelho V, Dernedde J, Petrausch U, Panjideh H, Fuchs H, Menzel C, Dubel S, Keilholz U, Thiel E, Deckert PM (2007) Design, construction, and in vitro analysis of A33scFv::CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer. Int J Oncol 31:951–957, PMID: 17786329
260. Biela BH, Khawli LA, Hu P, Epstein AL (2003) Chimeric TNT-3/human beta-glucuronidase fusion proteins for antibody-directed enzyme prodrug therapy (ADEPT). Cancer Biother Radiopharm 18:339–353. doi:10.1089/108497803322285099
261. Florent JC, Dong X, Gaudel G, Mitaku S, Monneret C, Gesson JP, Jacquesy JC, Mondon M, Renoux B, Andrianomenjanahary S, Michel S, Koch M, Tillequin F, Gerken M, Czech J, Straub R, Bosslet K (1998) Prodrugs of anthracyclines for use in antibody-directed enzyme prodrug therapy. J Med Chem 41:3572–3581. doi:10.1021/jm970589l
262. Haisma HJ, Sernee MF, Hooijberg E, Brakenhoff RH, Vd Meulen-Muileman IH, Pinedo HM, Boven E (1998) Construction and characterization of a fusion protein of single-chain anti-CD20 antibody and human beta-glucuronidase for antibody-directed enzyme prodrug therapy. Blood 92:184–190, PMID 9639515
263. Afshar S, Asai T, Morrison SL (2009) Humanized ADEPT comprised of an engineered human purine nucleoside phosphorylase and a tumor targeting peptide for treatment of cancer. Mol Cancer Ther 8:185–193. doi:10.1158/1535-7163.MCT-08-0652
264. Heinis C, Alessi P, Neri D (2004) Engineering a thermostable human prolyl endopeptidase for antibody-directed enzyme prodrug therapy. Biochemistry 43:6293–6303. doi:10.1021/bi0361160
265. Knox RJ, Friedlos F, Boland MP (1993) The bioactivation of CB 1954 and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT). Cancer Metastasis Rev 12:195–212. doi:10.1007/bf00689810
266. Lu JY, Lowe DA, Kennedy MD, Low PS (1999) Folate-targeted enzyme prodrug cancer therapy utilizing penicillin-V amidase and a doxorubicin prodrug. J Drug Target 7:43–53. doi:10.3109/10611869909085491
267. Padiolleau-Lefevre S, Naya RB, Shahsavarian MA, Friboulet A, Avalle B (2014) Catalytic antibodies and their applications in biotechnology: state of the art. Biotechnol Lett 36(7):1369–79. doi:10.1007/s10529-014-1503-8
268. Abraham S, Guo F, Li LS, Rader C, Liu C, Barbas CF 3rd, Lerner RA, Sinha SC (2007) Synthesis of the next-generation therapeutic antibodies that combine cell targeting and antibody-catalyzed prodrug activation. Proc Natl Acad Sci USA 104:5584–5589. doi:10.1073/pnas.0700223104
269. Zawilska JB, Wojcieszak J, Olejniczak AB (2013) Prodrugs: a challenge for the drug development. Pharmacol Rep 65:1–14. doi:10.1016/S1734-1140(13)70959-9
270. Jain S, Kumar D, Swarnakar NK, Thanki K (2012) Polyelectrolyte stabilized multilayered liposomes for oral delivery of paclitaxel. Biomaterials 33:6758–6768. doi:10.1016/j.biomaterials.2012.05.026
271. Sapra P, Shor B (2013) Monoclonal antibody-based therapies in cancer: advances and challenges. Pharmacol Ther 138:452–469. doi:10.1016/j.pharmthera.2013.03.004
272. Iden DL, Allen TM (2001) In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. Biochim Biophys Acta 1513:207–216. doi:10.1016/S0005-2736(01)00357-1
273. Ishida T, Iden DL, Allen TM (1999) A combinatorial approach to producing sterically stabilized (stealth) immunoliposomal drugs. FEBS Lett 460:129–133. doi:10.1016/S0014-5793(99)01320-4
274. Sofou S, Sgouros G (2008) Antibody-targeted liposomes in cancer therapy and imaging. Expert Opin Drug Deliv 5:189–204. doi:10.1517/17425247.5.2.189
275. Sapra P, Allen TM (2002) Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. Cancer Res 62:7190–7194, PMID: 12499256
276. Mastrobattista E, Koning GA, van Bloois L, Filipe AC, Jiskoot W, Storm G (2002) Functional characterization of an endosome-disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins. J Biol Chem 277:27135–27143. doi:10.1074/jbc.M200429200

277. Torchilin VP (2005) Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4:145–160. doi:10.1038/nrd1632
278. Gao J, Chen H, Yu Y, Song J, Song H, Su X, Li W, Tong X, Qian W, Wang H (2013) Inhibition of hepatocellular carcinoma growth using immunoliposomes for co-delivery of adriamycin and ribonucleotide reductase M2 siRNA. Biomaterials 34:10084–10098. doi:10.1016/j.biomaterials.2013.08.088
279. Gao J, Liu W, Xia Y, Li W, Sun J, Chen H, Li B, Zhang D, Qian W, Meng Y (2011) The promotion of siRNA delivery to breast cancer overexpressing epidermal growth factor receptor through anti-EGFR antibody conjugation by immunoliposomes. Biomaterials 32:3459–3470. doi:10.1016/j.biomaterials.2011.01.034
280. Deng L, Zhang Y, Ma L, Jing X, Ke X, Lian J, Zhao Q, Yan B, Zhang J, Yao J, Chen J (2013) Comparison of anti-EGFR-Fab′ conjugated immunoliposomes modified with two different conjugation linkers for siRNa delivery in SMMC-7721 cells. Int J Nanomedicine 8:3271–3283. doi:10.2147/IJN.S47597
281. Drummond DC, Noble CO, Guo Z, Hayes ME, Connolly-Ingram C, Gabriel BS, Hann B, Liu B, Park JW, Hong K, Benz CC, Marks JD, Kirpotin DB (2010) Development of a highly stable and targetable nanoliposomal formulation of topotecan. J Control Release 141:13–21. doi:10.1016/j.jconrel.2009.08.006
282. Nishikawa K, Asai T, Shigematsu H, Shimizu K, Kato H, Asano Y, Takashima S, Mekada E, Oku N, Minamino T (2012) Development of anti-HB-EGF immunoliposomes for the treatment of breast cancer. J Control Release 160:274–280. doi:10.1016/j.jconrel.2011.10.010
283. Apte A, Koren E, Koshkaryev A, Torchilin VP (2014) Doxorubicin in TAT peptide-modified multifunctional immunoliposomes demonstrates increased activity against both drug-sensitive and drug-resistant ovarian cancer models. Cancer Biol Ther 15:69–80. doi:10.4161/cbt.26609
284. Wicki A, Rochlitz C, Orleth A, Ritschard R, Albrecht I, Herrmann R, Christofori G, Mamot C (2012) Targeting tumor-associated endothelial cells: anti-VEGFR2 immunoliposomes mediate tumor vessel disruption and inhibit tumor growth. Clin Cancer Res 18:454–464. doi:10.1158/1078-0432.CCR-11-1102
285. Padhye SS, Guin S, Yao HP, Zhou YQ, Zhang R, Wang MH (2011) Sustained expression of the RON receptor tyrosine kinase by pancreatic cancer stem cells as a potential targeting moiety for antibody-directed chemotherapeutics. Mol Pharm 8:2310–2319. doi:10.1021/mp200193u
286. Guin S, Ma Q, Padhye S, Zhou YQ, Yao HP, Wang MH (2011) Targeting acute hypoxic cancer cells by doxorubicin-immunoliposomes directed by monoclonal antibodies specific to RON receptor tyrosine kinase. Cancer Chemother Pharmacol 67:1073–1083. doi:10.1007/s00280-010-1408-8
287. Mortensen JH, Jeppesen M, Pilgaard L, Agger R, Duroux M, Zachar V, Moos T (2013) Targeted antiepidermal growth factor receptor (cetuximab) immunoliposomes enhance cellular uptake in vitro and exhibit increased accumulation in an intracranial model of glioblastoma multiforme. J Drug Deliv 2013:209205. doi:10.1155/2013/209205
288. Lehtinen J, Raki M, Bergstrom KA, Uutela P, Lehtinen K, Hiltunen A, Pikkarainen J, Liang H, Pitkanen S, Maatta AM, Ketola RA, Yliperttula M, Wirth T, Urtti A (2012) Pre-targeting and direct immunotargeting of liposomal drug carriers to ovarian carcinoma. PLoS One 7:e41410. doi:10.1371/journal.pone.0041410
289. Hantel C, Lewrick F, Schneider S, Zwermann O, Perren A, Reincke M, Suss R, Beuschlein F (2010) Anti insulin-like growth factor I receptor immunoliposomes: a single formulation combining two anticancer treatments with enhanced therapeutic efficiency. J Clin Endocrinol Metab 95:943–952. doi:10.1210/jc.2009-1980
290. Koren E, Apte A, Jani A, Torchilin VP (2012) Multifunctional PEGylated 2C5-immunoliposomes containing pH-sensitive bonds and TAT peptide for enhanced tumor cell internalization and cytotoxicity. J Control Release 160:264–273. doi:10.1016/j.jconrel.2011.12.002

291. Kullberg M, Mann K, Anchordoquy TJ (2012) Targeting Her-2+ breast cancer cells with bleomycin immunoliposomes linked to LLO. Mol Pharm 9:2000–2008. doi:10.1021/mp300049n
292. Catania A, Barrajon-Catalan E, Nicolosi S, Cicirata F, Micol V (2013) Immunoliposome encapsulation increases cytotoxic activity and selectivity of curcumin and resveratrol against HER2 overexpressing human breast cancer cells. Breast Cancer Res Treat 141:55–65. doi:10.1007/s10549-013-2667-y
293. Yamamoto Y, Yoshida M, Sato M, Sato K, Kikuchi S, Sugishita H, Kuwabara J, Matsuno Y, Kojima Y, Morimoto M, Horiuchi A, Watanabe Y (2011) Feasibility of tailored, selective and effective anticancer chemotherapy by direct injection of docetaxel-loaded immunoliposomes into Her2/neu positive gastric tumor xenografts. Int J Oncol 38:33–39, PMID: 21109923
294. Srivastava A, O'Connor IB, Pandit A, Gerard Wall J (2014) Polymer-antibody fragment conjugates for biomedical applications. Prog Polym Sci 39:308–329. doi:10.1016/j.progpolymsci.2013.09.003
295. Hagemeyer CE, von Zur MC, von Elverfeldt D, Peter K (2009) Single-chain antibodies as diagnostic tools and therapeutic agents. Thromb Haemost 101:1012–1019, PMID: 19492141
296. Hu X, Spada S, White S, Hudson S, Magner E, Wall JG (2006) Adsorption and activity of a domoic acid binding antibody fragment on mesoporous silicates. J Phys Chem B 110:18703–18709. doi:10.1021/jp062423e
297. Albrecht H, Denardo GL, Denardo SJ (2006) Monospecific bivalent scFv-SH: effects of linker length and location of an engineered cysteine on production, antigen binding activity and free SH accessibility. J Immunol Methods 310:100–116. doi:10.1016/j.jim.2005.12.012
298. Weisser NE, Hall JC (2009) Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. Biotechnol Adv 27:502–520. doi:10.1016/j.biotechadv.2009.04.004
299. Yang K, Basu A, Wang M, Chintala R, Hsieh MC, Liu S, Hua J, Zhang Z, Zhou J, Li M, Phyu H, Petti G, Mendez M, Janjua H, Peng P, Longley C, Borowski V, Mehlig M, Filpula D (2003) Tailoring structure-function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation. Protein Eng 16:761–770. doi:10.1093/protein/gzg093
300. Kim SH, Lee YS, Hwang SY, Bae GW, Nho K, Kang SW, Kwak YG, Moon CS, Han YS, Kim TY, Kho WG (2007) Effects of PEGylated scFv antibodies against Plasmodium vivax duffy binding protein on the biological activity and stability in vitro. J Microbiol Biotechnol 17:1670–1674, PMID: 18156783
301. Kitamura K, Takahashi T, Takashina K, Yamaguchi T, Noguchi A, Tsurumi H, Toyokuni T, Hakomori S (1990) Polyethylene glycol modification of the monoclonal antibody A7 enhances its tumor localization. Biochem Biophys Res Commun 171:1387–1394. doi:10.1016/0006-291X(90)90839-F
302. Delgado C, Pedley RB, Herraez A, Boden R, Boden JA, Keep PA, Chester KA, Fisher D, Begent RH, Francis GE (1996) Enhanced tumour specificity of an anti-carcinoembryonic antigen Fab′ fragment by poly(ethylene glycol) (PEG) modification. Br J Cancer 73:175–182. doi:10.1038/bjc.1996.32
303. Casey J, Pedley R, King D, Boden R, Chapman A, Yarranton G, Begent R (1999) Improved tumour targeting of di-Fab′ fragments modified with polyethylene glycol. Tumor Target 4:235–244, http://discovery.ucl.ac.uk/333098/
304. Li L, Yazaki PJ, Anderson AL, Crow D, Colcher D, Wu AM, Williams LE, Wong JY, Raubitschek A, Shively JE (2006) Improved biodistribution and radioimmunoimaging with poly(ethylene glycol)-DOTA-conjugated anti-CEA diabody. Bioconjug Chem 17:68–76. doi:10.1021/bc0502614
305. Lu ZR, Kopeckova P, Kopecek J (1999) Polymerizable Fab′ antibody fragments for targeting of anticancer drugs. Nat Biotechnol 17:1101–1104. doi:10.1038/15085
306. Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S, Kerr DJ, Cancer Research Campaign Phase IIICTc (2002) Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin. J Clin Oncol 20:1668–1676, PMID: 11896118

307. Hovorka O, St'astny M, Etrych T, Subr V, Strohalm J, Ulbrich K, Rihova B (2002) Differences in the intracellular fate of free and polymer-bound doxorubicin. J Control Release 80:101–117. doi:10.1016/S0168-3659(02)00016-0
308. Satchi R, Connors TA, Duncan R (2001) PDEPT: polymer-directed enzyme prodrug therapy. I. HPMA copolymer-cathepsin B and PK1 as a model combination. Br J Cancer 85:1070–1076
309. Etrych T, Strohalm J, Kovar L, Kabesova M, Rihova B, Ulbrich K (2009) HPMA copolymer conjugates with reduced anti-CD20 antibody for cell-specific drug targeting. I. Synthesis and in vitro evaluation of binding efficacy and cytostatic activity. J Control Release 140:18–26
310. Gao J, Xia Y, Chen H, Yu Y, Song J, Li W, Qian W, Wang H, Dai J, Guo Y (2014) Polymer-lipid hybrid nanoparticles conjugated with anti-EGF receptor antibody for targeted drug delivery to hepatocellular carcinoma. Nanomedicine (Lond) 9:279–293. doi:10.2217/nnm.13.20
311. Khaw BA, Gada KS, Patil V, Panwar R, Mandapati S, Hatefi A, Majewski S, Weisenberger A (2014) Bispecific antibody complex pre-targeting and targeted delivery of polymer drug conjugates for imaging and therapy in dual human mammary cancer xenografts : targeted polymer drug conjugates for cancer diagnosis and therapy. Eur J Nucl Med Mol Imaging 41(8):1603–1616. doi:10.1007/s00259-014-2738-2
312. Liu J, Kopeckova P, Buhler P, Wolf P, Pan H, Bauer H, Elsasser-Beile U, Kopecek J (2009) Biorecognition and subcellular trafficking of HPMA copolymer-anti-PSMA antibody conjugates by prostate cancer cells. Mol Pharm 6:959–970. doi:10.1021/mp8002682
313. Satchi-Fainaro R, Wrasidlo W, Lode HN, Shabat D (2002) Synthesis and characterization of a catalytic antibody-HPMA copolymer-conjugate as a tool for tumor selective prodrug activation. Bioorg Med Chem 10:3023–3029. doi:10.1016/s0968-0896(02)00156-6
314. Berguig GY, Convertine AJ, Shi J, Palanca-Wessels MC, Duvall CL, Pun SH, Press OW, Stayton PS (2012) Intracellular delivery and trafficking dynamics of a lymphoma-targeting antibody-polymer conjugate. Mol Pharm 9:3506–3514. doi:10.1021/mp300338s
315. Tappertzhofen K, Metz VV, Hubo M, Barz M, Postina R, Jonuleit H, Zentel R (2013) Synthesis of maleimide-functionalyzed HPMA-copolymers and in vitro characterization of the aRAGE- and human immunoglobulin (huIgG)-polymer conjugates. Macromol Biosci 13:203–214. doi:10.1002/mabi.201200344
316. Rihova B, Kopeckova P, Strohalm J, Rossmann P, Vetvicka V, Kopecek J (1988) Antibody-directed affinity therapy applied to the immune system: in vivo effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody. Clin Immunol Immunopathol 46:100–114. doi:10.1016/0090-1229(88)90010-4
317. Merdan T, Callahan J, Petersen H, Kunath K, Bakowsky U, Kopeckova P, Kissel T, Kopecek J (2003) Pegylated polyethylenimine-Fab' antibody fragment conjugates for targeted gene delivery to human ovarian carcinoma cells. Bioconjug Chem 14:989–996. doi:10.1021/bc0340767
318. Pechar M, Ulbrich K, Jelínková M, Říhová B (2003) Conjugates of antibody-targeted PEG multiblock polymers with doxorubicin in cancer therapy. Macromol Biosci 3:364–372. doi:10.1002/mabi.200350004
319. Patel NR, Pattni BS, Abouzeid AH, Torchilin VP (2013) Nanopreparations to overcome multidrug resistance in cancer. Adv Drug Deliv Rev 65:1748–1762. doi:10.1016/j.addr.2013.08.004
320. Kirtane AR, Kalscheuer SM, Panyam J (2013) Exploiting nanotechnology to overcome tumor drug resistance: challenges and opportunities. Adv Drug Deliv Rev 65:1731–1747. doi:10.1016/j.addr.2013.09.001
321. Yan Y, Bjornmalm M, Caruso F (2013) Particle carriers for combating multidrug-resistant cancer. ACS Nano 7:9512–9517. doi:10.1021/nn405632s
322. Bertrand N, Wu J, Xu X, Kamaly N, Farokhzad OC (2014) Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. Adv Drug Deliv Rev 66C:2–25. doi:10.1016/j.addr.2013.11.009

Chapter 3
Infectious Diseases: Need for Targeted Drug Delivery

Padma V. Devarajan, Shilpa M. Dawre, and Rinku Dutta

3.1 Infectious Diseases in the Modern World

Infectious diseases are among the leading cause of death worldwide, even in the twenty-first century. Developing nations are more susceptible due to lack of proper sanitation, uneducated population and increasing pollution and the booming population explosion. Tuberculosis, HIV/AIDS, malaria are infectious diseases that have become epidemic in a true sense. According to a 2004 World Health Organization (WHO) report, infectious diseases are a major cause of morbidity in developing countries. A more recent report in 2012 records the death of more than 8.7 million people worldwide in 2008, due to infectious diseases. Diseases earlier confined to particular territories have changed face as global epidemics, due to globalisation and cross movement of people across geographical boundaries. A classic example is swine flu which originated in Asia and rapidly spread to the west.

3.1.1 Extracellular and Intracellular Infectious Diseases

Several microorganisms survive in the extracellular spaces within the body, or on epithelial surfaces, to cause extracellular infections. Extracellular pathogens release specific toxins or proteins which triggers the production of antibodies. On the other hand, intracellular infections reside within the cells of the body's defence system the reticuloendothelial system (RES). The normal body response to a pathogen is rapid opsonisation followed by phagocytosis, which results in killing and clearing

P.V. Devarajan (✉) • S.M. Dawre • R. Dutta
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (Elite status), Deemed University, Matunga, Mumbai, India
e-mail: pvdevarajan@gmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_3

Table 3.1 Infectious diseases and causative organisms

Intracellular diseases	
Infectious diseases	Causative organisms
AIDS/HIV	Human immunodeficiency virus
Cholera	*Vibrio cholerae* (bacteria)
Dengue	Dengue (RNA) virus
Hepatitis A/B/C	Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV)
Influenza	RNA viruses (Influenza A/B/C viruses)(e.g. H1N1)
Legionellosis	Legionella
Leishmaniasis	*Leishmania donovani*
Listeriosis	*Listeria monocytogenes*
Malaria	*Plasmodium* sp.
Shigellosis	*Shigella*
Tuberculosis	*Mycobacterium tuberculosis*
Typhoid	*Salmonella typhi*
Tularemia	*Francisella tularensis*
Extracellular diseases	
African trypanosomiasis	*Trypanosoma brucei gambiense, Trypanosoma brucei rhodesiense*
Pneumonia	*Streptococcus pneumonia, Haemophilus influenza, Chlamydophila pneumonia, Mycoplasma pneumonia, Staphylococcus aureus, Moraxella catarrhalis, Legionella pneumophila, Klebsiella pneumonia; rhinoviruses, coronaviruses, influenza virus, respiratory syncytial virus (RSV), adenovirus, and parainfluenza*
Schistosomiasis	*Schistosoma mansoni, Schistosoma intercalatum, Schistosoma haematobium, Schistosoma japonicum, Schistosoma mekongi*

of the microorganism. Intracellular infections result when the organisms cleverly evade destruction following phagocytosis. The intracellular location of these microorganisms protects them from the host defence mechanisms, such as antibodies or complement, and from the action of drugs that are unable to penetrate the cell efficiently. Hence, while adequate drug concentrations are readily achieved at extracellular infection sites to enable efficient therapy, intracellular infections are more difficult to treat. Some common intracellular and extracellular infectious diseases and their causative organisms are listed in Table 3.1.

3.2 Reticuloendothelial System and Intracellular Infections

The RES also known as the mononuclear phagocytic system (MPS)/macrophage system is the primary defence mechanism of the human body and hence the site of intracellular infections. The macrophages constitute the major defence cells of the RES. Derived from the bone marrow the RES also contributes to both non-specific and specific immunity. Recognition by the RES is facilitated by opsonins, with the step of opsonisation being a precursor to phagocytosis.

3.2.1 Opsonisation

Opsonisation is the process by which bacteria are altered by opsonins so as to become more readily and efficiently engulfed by phagocytosis. Opsonisation is mediated by the complement system: C3b, C4b, and iC3b, antibodies IgG and IgM and mannose-binding lectin. Mannose binding lectin initiates the formation of C3b. Opsonisation of particles enables recognition by the Fc receptors, complement receptors or specific receptors for phagocytosis. Opsonins are generally proteins which can bind to pattern-recognition receptors (PRRs) or other specific receptors expressed on the surface of macrophages. Pentraxins [C-reactive protein and serum amyloid P] [1], mindin, collectins [2] and ficolins [3] are such opsonins. The function of pattern-recognition receptors (PRRs) is to recognise and enhance phagocytosis of pathogen-associated molecular patterns (PAMPs), specific patterns present on microbial pathogens like lipopolysaccharide (LPS) in Gram-negative bacteria, lipotechoic acid (LTA) in Gram-positive bacteria and mannans in yeast. Toll-like receptors (TLRs) are PRRs essential for recognition of microbial components such as TLR4 (LPS) [4–6], TLR3 [double-stranded RNA] [7], TLR6 [mycoplasmal macrophage-activating lipopeptide—2 kDa] [8], TLR9 [CpG bacterial DNA] [9], TLR5 [bacterial flagellin] [10], and TLR2 [peptidoglycan]. However, the exact mechanisms of TLR recognition of microbial components remain unclear.

3.2.2 Phagocytosis

Opsonisation facilitates adherence of pathogens to macrophages, and is facilitated by integrins. Adherence induces membrane protrusions, called pseudopodia, to extend around the attached material. Following fusion with the macrophage, the pseudopodia forms a phagosome that encloses the pathogen within a membrane, which then enters the endocytic process. Phagosomes coalesce with intracellular organelles to mature into phagolysosomes, which have an acidic environment with many digestive proteins which finally degrades the internalised material. Phagocytised material is eliminated by exocytosis. The process of phagocytosis is mediated by several proteins such as actin, dynamin and cortactin. While actin is connected to the lipidic membrane and responsible for invagination of the membrane to form the endosome, cortactin is an actin-binding protein which stimulates its polymerisation. Dynamin hydrolyses guanidine triphosphate and uses the resulting energy for the contraction of actin and formation of endosome. Particulates that cannot be digested remain sequestered in residual bodies within the cell. Other cells such as fibroblast, endothelial and epithelial cells also exhibit phagocytic activity and can engulf microbes like Shigella, Listeria and Yersinia [11]. Such phagocytosis is mediated by laminin and fibronectin receptors/heparan sulfate present on the membrane surface [11]. However, the major cells responsible for phagocytosis are macrophages.

3.2.3 Macrophages

Macrophages (Greek: makros means large and phagein means eat) are cells formed by the differentiation of monocytes in tissues. Macrophages play an important role in both innate and adaptive immunity in vertebrates. These specialised phagocytic cells engulf and destroy infectious microbes, foreign particles and cancer cells [12]. The macrophages also regulate lymphocyte, granulocyte populations and important tumor growth modulators [13]. Macrophages act by both oxygen-dependent killing and oxygen independent killing mechanisms. The mediators for oxygen-dependent killing are reactive oxygen intermediates (ROIs) (superoxide anion, hydroxyl radicals, hydrogen peroxide and hypochlorite anion), reactive nitrogen intermediates (RNIs) (nitric oxide, nitrogen dioxide and nitrous acid) and monochloramine, while the mediators for oxygen independent killing are defensins, tumor necrosis factor (macrophage only), lysozyme and hydrolytic enzymes. Floating macrophages predominate in the vascular system, while tissue macrophages are localised in specific tissues. Based on the tissue of residence they have specific nomenclature (Fig. 3.1).

Macrophages can be classified mainly into two groups: (1) pro-inflammatory or classically activated macrophages (M1) and (2) anti-inflammatory or alternatively activated macrophages (M2).

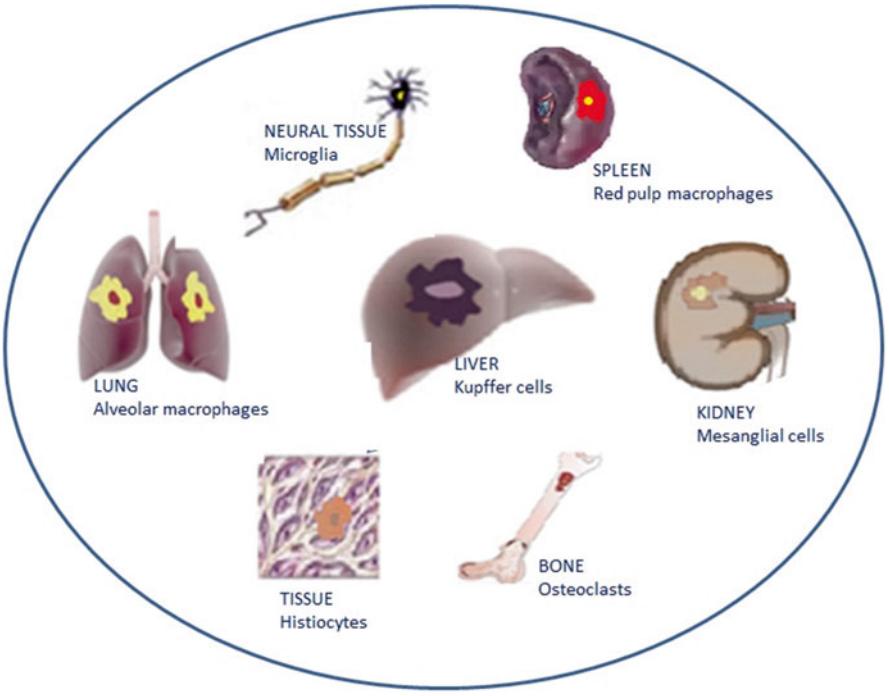

Fig. 3.1 Tissue macrophages and their organs of residence

3.2.3.1 Activated Macrophages (M1)

M1 macrophages are immune effector cells that aggressively work against microbes and cause their destruction much more readily. M1 is mainly associated with gastrointestinal infections (e.g. typhoid fever and *Helicobacter pylori* gastritis) and active tuberculosis. M1 macrophages are stimulated by interferon (IFN)-g or lipopolysaccharide (LPS) to release nitric oxide (NO), important for killing intracellular pathogens. Activated macrophages are characterised by expression of major histocompatibility molecule like MHC class II and CD86 and their ability to secrete proinflammatory cytokines such as tumor necrosis factor (TNF)-a, IL-1b, IL-12, IL-18 and the chemokines CCL15, CCL20, CXCL8-11 and CXCL13 [14]. Activated M1 macrophages facilitate killing of microorganisms by endocytosis, synthesising reactive oxygen intermediates (ROI), limiting the uptake of nutrients and iron essential for the growth of bacteria and replication of viruses, or production of nitric oxide facilitated by IFN-g-inducible NO synthase (iNOS).

3.2.3.2 Alternative Activated Macrophages (M2)

M2 macrophages are important for killing extracellular parasites, wound healing, tissue repair, and to turn-off immune system activation. M2 macrophages are activated by interleukin (IL)-4 or IL-13 (M2a) to produce IL-10, transforming growth factor (TGF)-b and arginase-1 (Arg1), to enable this function [14]. M2 macrophages are mostly observed in lepromatous leprosy, Whipple's disease and localised infections (keratitis, chronic rhinosinusitis).

A number of infectious organisms which manage to overcome the RES defence develop unique adaptive mechanisms which enable them to survive within the cell for prolonged periods of time. Eradication of such intracellular organisms poses immense challenges.

3.2.4 Survival Mechanisms Adapted by Pathogens

Many pathogens have an innate ability to develop adaptive mechanisms under stress conditions to fight for their survival. Such adaptive mechanisms or protective strategies, enables them to exhibit greater defence to the host and there by prolong survival. The different adaptive mechanisms employed by pathogens are discussed below.

3.2.4.1 Inhibition of Phagolysosome Formation

Strategies adopted by microorganisms to inhibit phagolysosome formation include interference with the transformation of primary endosomes into late endosome, fusion with lysosomes and or phagosome acidification. This delays the fusion of

Table 3.2 Mechanisms of inhibition of phagolysosome formation

Mechanism	Pathogens	Diseases	References
Enzymatic breakdown	*Mycobacterium tuberculosis*	Tuberculosis	Sturgill-Koszycki et al. [18]
	Mycobacterium leprae	Leprosy	Frehel and Rastogi [19]
	Listeria monocytogenes	Listeriosis	Alvarez-Dominguez et al. [20]
	Salmonella enteric	Salmonellosis	Buchmeier et al. [21]
	Leishmania spp.	Leishmaniasis	Desjardins et al. [22]; Mosser et al. [23]
	Toxoplasma gondii	Toxoplasmosis	Sibley [24]
	Helicobacter pylori	GIT infections	Borlace et al. [25]
	Trypanosoma cruzii	Trypanosomiasis	Ochatt et al. [26]
Lack of acidification	*Yersinia pestis*	Pneumonia, septicemia	Pujol et al. [17]
Disturbs the formation of lipid rafts by producing beta-1,2 glucans	*Brucella* spp.	Brucellosis	Roy [27]
Alteration of host cell signaling by dephosphorylation of signal regulated kinase	*Leishmania* spp.	Leishmaniasis	Ghosh et al. [28]

endosomes with lysosomes [15] or blocks the same [16]. The strategies to inhibit phagolysosome formation and the pathogens which exhibit the same [17] are summarised in Table 3.2.

3.2.4.2 Fusion of Endosome with Cell Organelles Other than Lysosome

Pathogens which exhibit this adaptation survive and multiply in vesicles formed by fusion of endosomes with cell organelles other than the lysosome, such as the rough endoplasmic reticulum, ribosome or mitochondria [29] and thus avoid phagolysosome formation. They thereby bypass destruction due to the enzymatic activity in the lysosome [30].

3.2.4.3 Disruption of the Phagolysosome

Escape from endocytosis is a crucial step for intramacrophagic survival. Pathogens from this category contain lytic enzymes which enable them to break the endosomes membrane and disrupt membrane of the vacuole [31], and hence evade degradation in the phagolysosome, and enter the cytosol rich in nutrients [32]. Specific enzymes are produced by the microorganisms for instance, *L. monocytogenes*

produces listeriolysin O (LLO) [33] and haemolysin C [34] while phospholipases are produced by the *Rickettsia* spp. [35].

3.2.4.4 Survival in the Late Phagolysosomes

The microbes in this category exhibit virulence factors which allow them to survive in lytic enzymes, acidic conditions and oxidants, the harsh conditions in the phagolysosome environment. Intramacrophagic resistance employing multiple virulence factors enables alternative pathways for survival and multiplication [36].

3.2.4.5 Internalisation by Non-phagocytic Pathways or by Parasitophorous Vacuole

Pathogens are internalised into macrophages by alternate routes. They traverse inside the cell by receptor mediated pathways like clathrin [37] and lipid rafts [38]. Formation of vesicles with new properties after fusion between the pathogen and membrane of the cell, like the parasitophorous vacuole formed by *Toxoplasma gondii* [38] also provides protection. In certain infections successful fusion of microorganisms with the macrophage is followed by secretion of antiapoptotic molecules (e.g. Bcl2). This results in impairment of apoptosis of the infected cells. Table 3.3 summarises illustrative examples of pathogens and their adaptive mechanisms for survival.

In addition to the adaptive mechanisms certain microbes employ highly specific strategies for persistence inside the cell. Such strategies are discussed with reference to some important diseases.

3.2.5 Specific Approaches of Some Important Pathogens for Persistence Inside the Cell

3.2.5.1 Tuberculosis

The adaptive mechanisms of *Mycobacterium tuberculosis* to survive inside the macrophages are prevention of fusion of the phagosome with lysosomes by producing tryptophan–aspartate-containing coat protein (TACO). Transformation of primary endosomes into phagolysosomes is prevented by a number of actions that occur simultaneously. These include reduced levels of proton ATPase inside the endosomes [18] removal of the Phosphatidylinositol 3-phosphate (PI3P) [16] and coupling of the inducible nitric oxide synthase (iNOS) [53]. The *M. tuberculosis* cell envelop comprises mycolic acid which can interact with cholesterol in the plasma membrane [50]. Further, mycobacteria are taken up inside macrophages by multiple receptors. The complement receptors are among the most widely used

Table 3.3 Other adaptive mechanisms of pathogens for persistence in macrophages

Adaptive mechanisms	Pathogens	Disease	References
Fusion of endosome with cell organelles other than lysosome	*Legionella pneumophila*	Legionellosis	Sibley et al. [38]
	Toxoplasma gondii	Toxoplasmosis	Sibley et al. [38]
Disruption of phagolysosome	*Listeria monocytogenes*	Listeriosis	Dabiri et al. [39]
	Shigella spp.	Shigellosis	Van der Wel et al. [40]
	Mycobacterium tuberculosis	Tuberculosis	Schroeder et al. [41]
	Mycobacterium leprae	Leprosy	Schroeder et al. [41]
	Francisella tularensis	Tularemia	Santic et al. [42]
	Trypanosoma cruzi	Trypanosomiasis	Andrews et al. [43]
	Rickettsia spp.	Typhus fever	Winkler et al. [44]
Survival in the late phagolysosomes	*Leishmania* spp.	Leishmaniasis	Alexander et al. [45]
	Legionella pneumophila	Legionellosis	Clemens et al. [46]
	Coxiella burnetii	Q fever	Burton et al. [47]
	Yersinia pestis	Pneumonia	Straley et al. [48]
	Staphylococcus aureus	Septicemic Endocarditis Bacteremia	Miller et al. [49]
Internalization by non-phagocytic pathways or parasitophorous vacuole	*Escherichia coli*	GIT infections	Shin et al. [37]
	Mycobacterium tuberculosis	Tuberculosis	Gatfield et al. [50]
	Salmonella spp.	Salmonellosis	Catron et al. [51]
	Clostridium spp.	Q fever	Simons et al. [52]
	Streptococcus spp.	Meningitis, bacterial pneumonia, endocarditis	Simons et al. [52]
	Toxoplasma gondii	Toxoplasmosis	Sibley et al. [38]

receptors for mycobacteria, for both opsonised and non-opsonised entry [54–56]. Other receptors are mannose receptors that bind glycosylated structures on the bacterial surface [57]. Fc receptors that can internalise IgG-opsonised bacteria [58] and scavenger receptors [59, 60] have also been implicated in mycobacterial uptake. Uptake of mycobacteria by the complement receptor pathway protects it from the aggressive lysosomal compartment ensuring relatively hospitable conditions.

3.2.5.2 Salmonellosis

Salmonella specifically forms a glycolipid capsule or biofilm. Biofilm formation in salmonella is related to the multicellular and aggregative response of rdar [61], rugose [62], or lacy [63]. This multicellular behavior is a property of salmonellae [64]

and is responsible for elaboration of thin fimbriae like Tafi, curli [65], cellulose [66], and other uncharacterised extracellular polysaccharides. Together, these components form the extracellular matrix that confers resistance to acid and bleach and facilitates environmental persistence [62, 64, 67–70].

3.2.5.3 Fungal Infections

Pathogens which cause fungal infections adapt various mechanisms to increase their pathogenesis and survive inside macrophages. *C. albicans* contains superoxide dismutases (SOD) and catalase enzymes which are able to convert O^{2-} into molecular oxygen and hydrogen peroxide, thereby decreasing the scavenging and toxic effects of O^{2-} and H_2O_2 levels by certain reactions [71]. Further, *C. neoformans* evade phagocytic uptake by phenotypic switching. This mechanism is observed in yeast cells that express glucuronoxylomannan mucoid capsule that resist phagocytic uptake and cause high lethality in mice [72]. In case of *Aspergillus conidia* infection collectins, pentraxin proteins are essential for opsonisation, but their deficiency is responsible for high susceptibility to infection in immunocompetent mice. Furthermore, several enzymes such as elastases and proteases released by the fungus enable conidia to escape from phagocytic uptake by alveolar macrophages.

3.2.5.4 HIV Infection

In HIV-1-infected macrophages, the viral envelope protein induces macrophage colony-stimulating factor (M-CSF). This pro-survival cytokine down regulates the TRAIL (tumor necrosis factor-related apoptosis-inducing ligands) receptor and up regulates the anti-apoptotic genes Bfl-1 and Mcl-1 enabling HIV to survive inside the macrophages. HIV invades the macrophage through CCR5 a chemokine receptor and through binding of gp120 to CD4 [73]. Macropinocytosis as a route of entry of HIV-1 into macrophages [74] also enables intracellular protection.

3.2.5.5 Leishmaniasis

Leshmania prevent activation of macrophages by inhibiting secretion of cytokines such as the inflammatory response IL-1 and tumor necrosis factor beta (TNF-beta) or T-lymphocyte activation (IL-12) and produce various immunosuppressive signaling molecules, such as arachidonic acid metabolites and the cytokines TNF-beta and IL-10. *L. chagasi* induces TNF-beta production in the immediate environment of the infected human macrophage, and this may lead to inhibition of immune responses [75]. Further, this pathogen induces alteration of host cell signaling. Macrophages infected with *L. donovani* or *L. mexicana* have shown altered Ca^{2+} dependent responses, such as chemotaxis and production of ROI [76, 77].

3.3 Intracellular Targets

Based on the adaptive mechanisms microorganisms reside in different cells and at different locations in the cells. Treating diseases therefore, necessitates an understanding of both the resident cells and target organelles. Illustrative examples of microorganism and their cellular/organelles targets are listed out in Table 3.4.

3.4 Other Reticuloendothelial System Cells

The granulocytes are classified as neutrophils, eosinophils, or basophils on the basis of cellular morphology. Neutrophils play the major role in the body's defence.

3.4.1 Neutrophils

Neutrophils are produced in the bone marrow by hematopoiesis. They are released into blood where they circulate for 7–10 h and migrate into tissues where they have a life span of a few days. During infection the bone marrow releases more than usual

Table 3.4 Diseases and intracellular targets of pathogens

Intracellular diseases	Target Cell	Target organelle	References
AIDS/HIV	T cells, epithelial cells	Phagosome, nucleus	D'Orsogna [78]
Brucellosis	Macrophage	Phagosome/lysosome or vacuole, endoplasmic reticulum	Roop [79]; Celli [80]
Dengue	WBCs, hepatocytes, vascular endothelial cells	–	Libraty et al. [81]
Hepatitis B, C	Hepatocytes	Endoplasmic reticulum	Moradpour [82]
Herpes Simplex virus (HSV-2)	Epithelial cells, neural ganglion	Nucleus	Heinz et al. [83]
Influenza	Respiratory epithelial cells	–	Arnheiter et al. [84]
Legionellosis	Macrophages	Phagosome/lysosome or vacuole, endoplasmic reticulum	Tilney et al. [85]
Leishmaniasis	Macrophages	–	Handman et al. [86]
Listeriosis	Macrophages	Cytosol	Collins, [87]
Malaria	Hepatocytes, red blood cells	–	Moulder [88]
Salmonella infection	Macrophages	Phagosome/lysosome or vacuole	Trebichavsky [89]
Tuberculosis	Alveolar macrophages, dendritic cells	Phagosome/lysosome or vacuole	Skvortsov [90]
Tularemia	–	Cytosol	Al-Khodor [91]

number of neutrophils, which migrate to the site of the infection. They act by both oxygen-dependent and oxygen-independent pathways to kill microbes. Neutrophils exhibit a larger respiratory burst than macrophages and consequently are able to generate more reactive oxygen intermediates and reactive nitrogen intermediates. In addition, neutrophils express higher levels of defensins than macrophages do. Hence, neutrophils are more active than macrophages in killing ingested microorganisms.

3.4.2 Dendritic Cells

Dendritic cells are antigen-presenting cells and constitute 0.5–1 % of the leukocyte population in the peripheral blood mononuclear cells. They are found mostly in non-lymphoid tissues and organs such as skin, heart, liver, lungs, and mucosal surfaces. The function of these cells is to initiate, stimulate and regulate a T cell response which includes antigen-specific T lymphocytes, Th1/Th2 modulation, regulatory T cell induction and peripheral T cell deletion. There are four types of dendritic cells, i.e. Langerhans cells, myeloid dendritic cells, plasmacytoid dendritic cells and infiltrating inflammatory dendritic epidermal cells. CD1b, CD11a, CD11b and CD11c, the thrombospondin receptor (CD36), and the mannose receptor (CD206), present on inflammatory dendritic epidermal cells, are known to be involved in the uptake of bacterial components. In case of *Mycobacterium tuberculosis* infection, alveolar macrophages (dust cells), along with dendritic cells engulf bacteria and exhibit innate as well as an adaptive immune response. Combined efforts by macrophages and dendritic cells establish protective immunity in 90 % of infected individuals.

3.4.3 Natural Killer Cells

Natural killer cells (NKC) are non-phagocytic cells present mostly in mammalian and avian species [92]. NKC express surface receptors for the Fc portion of IgG and their function is to mediate antibody-dependent cytotoxicity against tumor target cells [93]. It is also suggested that NKC play a role in resistance against some microbial infections. NKC also play a role in natural genetic resistance to infections caused by *cytomegalovirus* and *herpes simplex type I* [94, 95]. However, there is also evidence against the role of NKC in resistance to some other viruses [96].

3.4.4 Lymphoid Cells

Lymphocytes are cells present 99 % in the lymph and constitute 20–40 % of the body's white blood cells. There are approximately ~10^{10}–10^{12} lymphocytes in the human body, and this can vary with body weight and age. They circulate in the lymph and

blood, and can migrate into tissue spaces and lymphoid organs, enabling integration with the immune system. The two main categories of lymphoid cells that can recognise and react against a wide range of specific antigens are B lymphocytes or B cells and T lymphocytes or T cells.

3.4.4.1 B Lymphocytes

The main function of B cells is to produce antibodies against antigens [97]. Each of the approximately 1.5×10^5 molecules of the antibody on the membrane of a single B cell has identical binding sites for antigen. B cells express various receptors on the surface and exhibit following function for instance, Class II MHC molecules permit the B cell to function as an antigen-presenting cell (APC), CR1 (CD35) and CR2 (CD21) are receptors for certain complement products, while the FcRII (CD32) is a receptor for IgG, a type of antibody. Interaction of the membrane-bound antibody present on mature B cells with the antigen, as well as the interactions of the antigen with macrophages and T cells, results in B-cell clones of corresponding specificity. Repeated division of the B cell over 4–5 days generates a population of memory cells and plasma cells. Further plasma cells, are responsible for synthesis and secretion of antibody.

3.4.4.2 T Lymphocytes

Natural T lymphocytes mature in the thymus region and survive in the periphery. The chief function of T cells is to respond to signals associated with tissue destruction and to minimise the collateral tissue damage they cause [98]. T cells express T-cell receptors (TCR) which are a composite of polypeptides including CD3 and either of one of the two membrane molecules, CD8 and CD4. TCR recognises virus infected cells and cancer cells. However, unlike B cells, TCR does not recognise free antigen, unless it is bound to MHC molecules on the membrane of antigen presenting cells. The main function of T cells is to induce death of virus infected cells by secretion of cytotoxins and cytokines which activates B cells, macrophages and cytotoxic T cells. T cells also play role in infectious diseases such as Leishmaniasis [99], infection by hepatitis C virus (HCV), etc. Their ability to confine exuberant immune reactivity, associated with many chronic infections is beneficial the host due to limited tissue damage [100].

3.5 Non-specific Immune System Cells

Infectious diseases are also located in cells other than cells of the RES. Such cells include hepatocytes, epithelial cells and erythrocytes. Hepatocytes are located in the liver and are major site for infections such as hepatitis B/C and malaria.

The hepatocytes are discussed in greater detail in Chapter 6 of this book. Epithelial cells bind together to form the epithelial tissue which is held together by adherens, tight junctions, gap junctions and desmosomes. The functions of epithelial cells are boundary and protection of vital organs, transportation, absorption, secretion, lubrication and movement. These epithelial cells can be readily attacked by microbes such as HIV virus, influenza, Herpes Simplex virus (HSV-2) and cause infections. Furthermore, erythrocytes are infected and act as hosts for plasmodium causing malaria, one of the current fatal infections posing serious challenges.

3.6 Limitations of Conventional Therapy for Infectious Diseases

The introduction of antimicrobial agents such as penicillin resulted in a major breakthrough to decrease morbidity and mortality caused by infectious diseases. Antibiotics represented one of the greatest discoveries. This euphoria was short lived due to adverse effects and the emergence of drug resistance. Conventional therapy when associated with side effects or necessitates long term treatment, results in low patient compliance. Further inadequate drug concentration within cells is a major barrier for effective treatment of intracellular diseases. Increasing the dose, however, resulted in enhanced toxicity. Mono-drug therapy evolved into multi-drug therapy, and enabled a good degree of success and continues to form standard therapy, even today. Classic examples include the multi-drug combination for tuberculosis AKT2, AKT3, AKT4 comprising 2, 3 or 4 drugs, respectively. The HAART combination for AIDS and two drug combinations for malaria are also examples of successful therapy. Nevertheless, the alarming rate at which drug resistance is occurring, and more so the emergence of multi-drug resistance are a matter of great concern. Tuberculosis is one such major disease which has evolved from Resistant to Multi-drug Resistant(MDR) to total drug resistant (TDR), the latest being extremely drug resistant tuberculosis (XXDR), wherein, resistance is seen to almost all known antitubercular drugs.

3.6.1 Multi-drug Resistance (MDR)

The emergence of multi-drug resistance is attributed to a number of factors. Pathogens resort to different mechanisms to avoid intracellular killing. Some pathogens secrete exotoxins which destroy phagocytes and prevent phagocytosis. Bacteria with pore forming cytolysins avoid the phagosome and also escape lysosomal destruction [101–105]. Certain bacteria interfere with the production of cytotoxic metabolites of phagocytes or contain the antioxidant proteins, thereby overcoming the effects of RNIs or ROIs and cause obstruction in phagocytosis [106, 107].

3.6.2 Microbial Biofilms

Bacteria adhere to surfaces, aggregate and form a hydrated polymeric matrix comprising of exopolysaccharide known as biofilms [108]. Biofilms are developed by various bacteria such as *Salmonella*, *Streptococcus, Vibrio cholerae, Klebsiella pneumonia and Haemophilus influenzae.* Further some cells in the biofilm experience nutrient limitation and therefore survive in the starved state. Such cells are slow growing cells and less susceptible to antimicrobial agents [109]. Certain cells in a biofilm adapt a different and protected phenotype. Biofilms are resistant to antibodies, phagocytes, and antibiotics. Although *p*hagocytes reach the biofilms, they become frustrated and release their enzymes, which cause damage to the tissue around the biofilm. Release of bacteria through the damaged biofilm results in dissemination of the infection, leading to acute infection in the surrounding tissues [110, 111].

3.6.3 Efflux Pumps

Efflux pump genes and proteins are present in almost all organisms. Efflux pumps thwart the entry of an antibiotic in the bacterial cell and export an antibiotic from the cell. As efflux pumps can be specific for one substrate or for drugs of dissimilar structure, they can be associated with multi-drug resistance. Multi-drug-resistance efflux pumps are a known cause for the development of bacterial resistance against antibiotics. Bacterial efflux-pump proteins related with MDR are divided into five families namely *the ATP binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multi-drug and toxic-compound extrusion (MATE) family, the small multi-drug resistance (SMR) family and the resistance nodulation division (RND) family* [112]. Multi-drug resistance occurs, when efflux proteins are overexpressed on the cell, and easily identify and efficiently expel a broad range of antibiotics from the cells [113]. Gram-negative bacteria express several families of transporters which cause resistance [114]. Gram-positive bacteria mainly *Staphylococcus aureus* and *Streptococcus pneumoniae* express MDR efflux pumps. *S. aureus* (responsible for skin and soft-tissue infections) overexpress MFS efflux pump NorA which enables resistance to chloramphenicol and fluoroquinolones. The *S. pneumoniae* MFS efflux pumpPmrA exports the fluoroquinolones ciprofloxacin, norfloxacin, and also expels the dyes acriflavine and ethidium bromide [115–117] *Escherichia coli* EmrE express a member of the small multi-drug resistance (SMR) superfamily and AcrAB–TolC, a member of the resistance-nodulation-cell division (RND) superfamily. *Vibrio parahaemolyticus* overexpress NorM, a member of the multi-drug and toxic compound extrusion (MATE) superfamily.

Multi-drug-resistant tuberculosis (MDR-TB) is appearing as a ghost among the MDR bacteria because TB patients are at high risk of death due to failure of treatment. It is evident that MDR exhibits p55 efflux pumps which play a crucial

role in the pathogenicity of the microorganisms, and is responsible for the efflux of tetracycline and aminoglycosides. This has opened a vast array for research in identifying mutants which are responsible for overexpressing these protein pumps in cases of elevated virulence [112].

3.6.4 Enzymatic Drug Degradation and Chemical Modification

Chemical modification of antibiotics resulting in their inactivation and hence, ineffective dug concentration can be a cause of bacterial resistance. The inactivation reactions include hydrolysis, redox, and group transfer. Hydrolysis is the major cause of degradation of beta lactam antibiotics. The group transfer approach is the most varied and includes modification by thiol transfer, glycosylation, acyl transfer, ribosylation, nucleotidylation and phosphorylation transfer. Drugs which are degraded by group transfer are aminoglycoside, chloramphenicol, rifamycin, macrolides, etc. [118].

3.7 Strategies to Overcome Limitations of Conventional Drug Delivery

One important strategy to overcome the limitation of conventional drug delivery is to deliver high therapeutic payloads intracellularly. This could ensure high efficacy, coupled with low toxicity to provide major advantages. Targeted nanocarriers provide high promise as potential drug delivery systems with the capacity to address this specific challenge. Targeted nanocarriers could therefore prove to be the magic wand.

Passive and active targeting approaches could be relied on to achieve organ based targeting (first order), specific cell based targeting in an organ (second order) and cell organelle based targeting (third order) [119]. A major requirement, however, besides reaching the targeting site is to ensure adequate concentration and adequate retention at the site.

3.7.1 Passive Targeting

Passive targeting can be described as deposition of drug or drug-carrier systems at a particular location due to pharmacological or physicochemical factors [120]. Passive targeting can be achieved by exploiting pathophysiological and anatomical opportunities. Introduction of drugs directly into various anatomical sites for example

lungs and the eye by using non-invasive or invasive methods such as catheters or direct injections can enable local targeting. These site specific drug delivery methods limit systemic toxicity of the drug thus reducing adverse effects of drugs in the non-target tissues [121]. Exploiting altered pathological conditions in diseased tissues are strategies that can be adopted for passive targeting for example chemotactic factors released in infected or inflamed tissues increased permeability of vascular tissues, decreased pH and/or increased temperature [122, 123]. Increased vascular permeability specifically in cancers has enabled passive targeting of nanocarriers and is cited as the enhanced permeation and retention effect (EPR) effect [124]. Surface properties such as particle size, shape, hydrophobicity and surface charge have great impact on macrophage activation and phagocytosis.

3.7.1.1 Size

Particle size plays essential role in distribution and elimination of nanocarriers [125]. Particles size can influence attachment, adhesion, phagocytosis, distribution, circulation half-life and endocytic pathways [126, 127]. The opsonisation and phagocytosis of particles is strongly affected by size of nanocarriers. Although macrophages engulfed 0.2 versus 2 μm IgG-coated spherical particles by different mechanisms, they followed similar kinetics [clathrin endocytosis versus Fc-receptor mediated phagocytosis]. Phagocytic uptake is generally observed with polymeric particles and liposomes with high particle size [>200-microns] [128]. Table 3.7 highlights the size of a number of nanocarriers evaluated for targeted delivery in infectious diseases.

3.7.1.2 Shape

A broad range of non-spherical shaped particles studied including cylinders, cubes, hemispheres, ellipsoids, cones and complex shapes like filamentous, biconcave discoid showed varying effects on phagocytosis [169]. Non-spherical shaped particles bypassed phagocytosis due to incomplete actin structure formation. Particle shape affected attachment and internalisation during phagocytosis [170]. For instance oblate ellipsoids show best attachment and internalisation by phagocytosis, while prolate ellipsoids showed good attachment but poor internalisation. Champion et al. reported that worm-like particles showed low phagocytosis as compared to spherical particles of the same volume [169]. Asymmetric polymer lipid nanostructures (LIPOMER) of Doxycycline hydrochloride (DH) in the range of (250–400 nm) [171] revealed enhanced splenic delivery. The irregular shape of the LIPOMER coupled with rigidity resulted in filtration and non-phagocytic accumulation to reveal splenotropy in sinusoidal spleen models, rat, rabbit and dog. A high spleen liver ratio of 6.7:0.53 was seen in the dog model (Fig. 3.2) [172].

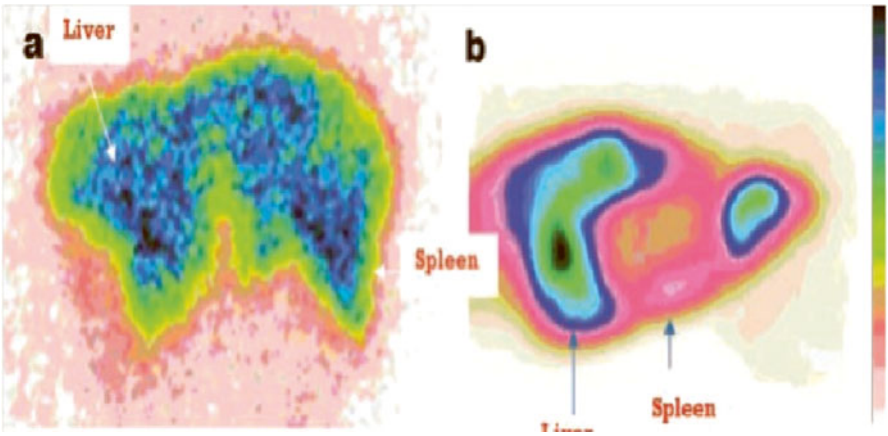

Fig. 3.2 Gamma scintigraphic images—biodistribution of LIPOMER in dog: (**a**) irregular, (**b**) spherical. Reprinted with permission from John Wiley and Sons

3.7.1.3 Surface Properties

Surface properties like hydrophobicity and surface charge also impact opsonisation, phagocytosis and biodistribution of nanoparticles [173]. Hydrophobic nanocarriers are readily coated by complement proteins, albumin, and immunoglobulin and scavenged by RES [174]. Surface charge of particles also influences interaction and stability with cells [175]. Reports suggest that positively charged particles showed high phagocytic uptake over negatively charged particles probably due to better interaction with the negatively charged cell membrane. Cationic and neutral nanocarriers are less taken up by RES as compare to negatively charge [176–178]. However, negatively charged nanoparticles can potentially attach to cationic sites on the macrophages namely the scavenger receptors, which facilitate their uptake by RES [179].

For details on influence of particle size, shape and charge readers are directed to the following reviews [126, 180].

3.7.2 Active Targeting

Active targeting, defined as specific targeting of drugs or drug containing nanocarriers by anchoring active agents or ligands, provides selectivity, recognisability and potential to interact with specific cells and tissues in the body [181]. Targeting by attaching ligands has been investigated as an additional strategy to enhance translocation of antimicrobials inside cells. Attaching ligands facilitates greater uptake and can be mediated by various mechanisms.

3.7.3 *Receptor Mediated Endocytosis (RME)*

The membrane of macrophages expresses various receptors to facilitate the internalisation of cargoes inside the cell and their degradation. Receptor mediated endocytosis (RME) permits the rapid internalisation of ligand attached particles as compared to untargeted particles [182]. The common RME mechanisms are macropinocytosis, clathrin dependent endocytosis (CDE), caveolae-mediated endocytosis and clathrin independent endocytosis (CIE). Each approach exhibits different binding and internalisation mechanisms. Further, the predominant uptake mechanism is often dictated by the nature of the ligand. Receptor mediated processes are relatively slower than phagocytic processes, with the ligand playing an important role. The sizes, geometry, charge and density of the ligand significantly influences receptor mediated endocytosis [183]. For more references readers can refer to [182, 184, 185]. Table 3.5 lists the endocytic pathways, endosome morphology and the proteins involved in the endocytic pathways.

Table 3.5 Endocytic pathways, endosome morphology and the proteins involved in the endocytic pathways

Endocytic mechanism	Proteins	Morphology	References
Clathrin mediated endocytosis	Dynamin, AP180, adaptin, Clathrin, AP2, epsin, SNX9, synaptojanin, actin, amphiphysin, Rab5, Arf6 plus many others	Vesicular	Ford et al. [186]; Roth et al. [187]
Caveolae mediated endocytosis	Caveolins, Cavins, PTRF, src, PKC, actin	Vesicular/ tubulovesicular	Parton et al. [188]; Rothberg et al. [189]; Krajewska et al. [190]
Flotillin-dependent endocytosis	Flottilin-1 and -2	Vesicular	Glebov et al. [191]; Frick et al. [192]
Clathrin-independent carrier (CLIC)/GPI-AP-enriched early endosomal compartment (GEEC)	ARHGAP10, actin, GRAF1, other GRAFs, Cdc42, Arf1	Tubular/ring	Lundmark et al. [193]; Naslavsky et al. [194]
ADP-ribosylation factor 6 (Arf6) mediated CIE	Arf6	Vesicular/ tubular	Donaldson et al. [195]
Macropinocytosis	Phosphoinositide 3-kinase, Rac1, Brefeldin A-ADP ribosylated substrate (BARS), Actin, PAK1, PI3K,Ras, Src, HDAC6	Highly ruffled	Kirkham et al. [196]; Marbet et al. [197]
Circular dorsal ruffle	Cortactin, actin	Highly ruffled	Krueger et al. [198]
IL2Rβ pathway	RhoA, Rac1, PAK1, PAK2	Vesicular?	Grassart et al. [199]; Lamaze et al. [200]

Table 3.6 Receptors expressed by macrophages and their specific ligands

Receptor	Ligands	References
Mannose	Mannose, fucose, N-acetyl glucosamine, glucose, collagen, mannan, mannosyl lipoarabinomanan	Ezekowitz et al. [201]
Tuftsin	Tuftsin	Agrawal and Gupta [202]; Tzehoval et al. [203]
Scavenger	Modified LDL, lipopolysaccharides (LPS), lipoteichoic acid (LTA)	Wilkinson and Khoury [204]; Graversen et al. [205]
Fc	Monoclonal Antibody	Guilliams et al. [206]
Fibronectin	Fibronectin, laminin, serum amyloid P	Taylor et al. [207]; Schett 2008
Folate	Folic acid	Kroger et al. [208]; Van Der Heijden et al. [209]
Transferrin	Transferrin	Qian et al. [210]
Toll-like receptor	Lipopolysaccharides (LPS), lipoproteins, lipopeptides and lipoarabinomannan	Kawai and Akira [211]
Complement receptors CR3 and CR4	C3b, iC3b, C3c and C3d	Campagne et al. [212]

3.7.4 Receptors for Macrophage Targeting

Macrophages possess large number of surface receptors which help in the process of recognition and endocytosis of engineered particulate carriers. Infection of macrophages leads to changes in the expression pattern of the concerned receptors, which can be exploited for targeted drug delivery employing nanocarriers. Table 3.6 is a summary of the important receptors on macrophages and illustrative examples of ligands for the same that could play a role in designing targeted nanocarriers for infectious disease therapy.

CD14 [213], Decay accelerating factor (CD55), Endo180 [214] are also receptors which could be targeted. Nevertheless, ligands for the same need to be explored.

3.8 Nanocarriers for Targeted Delivery in Infectious Diseases

All known nanocarriers can be effectively employed for targeted delivery in intracellular infections. Both passive and active targeting approaches have been evaluated. The following Tables 3.7 and 3.8 illustrate examples of nanocarriers, limited to major anti-infective agents for active and passive targeting, respectively. Size being a major parameter influencing targeting to RES. Table 3.7 also highlights the size of nanocarriers, which is a primary factor in passive targeting.

Table 3.7 Nanocarriers containing anti-infective agents and their particle size for passive targeting

Nanocarriers	Drug	Particle size	Diseases	References
	Polymyxin B	343 ± 28 nm	*Pseudomonas aeruginosa*	Alipour et al. [129]
Liposomes	Clofazimine	–	Tuberculosis	Mehta et al. [130]
	Pyrazinamide and rifabutin	0.1 μm	Tuberculosis	El-Ridy et al. [131]; Gaspar et al. [132]
	Ampicillin	208 + 70 nm	Salmonellosis	Fattal et al. [133]
	Gentamicin and streptomycin	–	Brucellosis	Fountain et al. [134]
	Ciprofloxacin	–	Salmonellosis	Magallanes et al. [135]
	Antimonials	–	Leishmaniasis	Date et al. [136]
	Dideoxycytidine	0.3 μm	HIV	Oussoren et al. [137]
Polymeric nanoparticles	Rifampicin gelatin NPS	264 ± 11.2 nm	Tuberculosis	Saraogia et al. [138]
	Guar gum	895.5 ± 14.73 nm	Tuberculosis	Kaur et al. [139]
	Rifampicin and isoniazid	382 ± 23 nm	Tuberculosis	Booysen et al. [140]
	Rifampicin	200-260 ± 10.24 nm	Tuberculosis	Esmaeili et al. [141]
	Indinavir	1.6 μm	HIV-1 encephalitis (HIVE)	Dou et al. [142]
	Rifampin and azithromycin antibodies	260 nm	Chlamydia infection	Toti et al. [143]
	AMB	250 nm	Leishmaniasis	Tyagi et al. [144]
	Gentamicin	245 ± 45 nm	Leishmaniasis	Zhang et al. [145]
	Rifampicin	–	*Staphylococcus aureus* and *Mycobacterium avium*	Azrami et al. [146]
	Moxifloxacin	418 ± 90.2 nm	Tuberculosis	Kisich et al. [147]
	Quinine	176 nm	Malaria	Hass et al. [148]
	AMB	358 ± 62 nm	Leishmaniasis	Espuelas et al. [149]
Gelatin NPS	Rifampicin	264 ± 11.2 nm	Tuberculosis	Saraogi et al. [138]
Microparticles	Isoniazid and rifabutin	–	Tuberculosis	Yadav et al. [150]
	Isoniazid	1 μm	Tuberculosis	Zhou et al. [151]
Solid lipid nanoparticles	Lopinavir	230 nm	HIV	Alex et al. [152]
	Tobramycin	855 nm	Bacterial	Bargoni et al. [153]
	Zidovudine	294 + 32 nm	HIV	Heiati et al. [154]
	Atazanavir	167 nm	HIV	Chattopadhay et al. [155]
	Isoniazid	131.7 nm	Tuberculosis	Bhandari and Kaur [156]

(continued)

Table 3.7 (continued)

Nanocarriers	Drug	Particle size	Diseases	References
Metallic nanoparticles	Rifampin, Isoniazid	100 nm	Tuberculosis	Clemens et al. [157]
Niosomes	Isoniazid	450 nm	Tuberculosis	Singh et al. [158]
Nanoemulsions/ nanosuspension	Primaquine	10–200 nm	*Plasmodium berghei*	Singh et al. [159]
	AMB	–	Leishmaniasis	Falk et al. [160]
Dendrimer	Lamivudine	–	HIV	Dutta and Jain [161]
	Primaquine phosphate	–	Malaria	Bhadra et al. [162]
Carbon nanotubes	AMB	100–400 nm	Leishmaniasis	Prajapati et al. [163]
Cu oxide		20–95 nm	*Meticillin-resistant Staphylococcus aureus (MRSA); Escherichia coli (E.coli)*	Ren et al. [164]; Raffi et al. [165]
Zn oxide		50–70 nm	*Staphylococcus aureus*	Jones et al. [166, 167]
Iron nanoparticles		3–9 nm	*E. coli*	Chatterjee et al. [168]

Table 3.8 Nanocarriers containing anti-infective agents and ligands for active targeting

Nanocarriers	Targeting Ligands	Drug	Diseases	References
Liposomes	Mannose	Pentamidine isethionate	Pneumocystis pneumonia	Banerjee et al. [215]
	Mannose	Ciprofloxacin	Respiratory intracellular parasitic infections	Chono et al. [216]
	Hyaluronan	Anti-inflammatory drug	Inflammatory sites	Glucksam-Galnoy et al. [217]
	Apolipoprotein E	–	Hepatic diseases	Kim et al. [218]
	Polyinosinic acid and phosphatidylserine	Antimony-lipopolysaccharide (Sb-LP)	Leishmaniasis	Tempone et al. [219]
	Ostearoylamylopectin (O-SAP)	Rifampicin and Isoniazid	Tuberculosis	Deol et al. [220]
	O-palmitoyl amylopectin (OPA)	Amphotericin B	Pulmonary candidiasis	Vyas et al. [221]
	IgG	–	Liver disease	Derksen et al. [222]

(continued)

Table 3.8 (continued)

Nanocarriers	Targeting Ligands	Drug	Diseases	References
	Tuftin	AMB	Leishmaniasis	Agrawal et al. [202]
	Immunoliposomal	AMB	HIV-1	Bestman-Smith et al. [223]
	Antibodies against human and murine HLA-DR and CD4 antigen	Indinavir	HIV	Gangne et al. [224]
Nanoparticle	Mannose	Rifampicin	Visceral leishmaniasis	Chaubey et al. [225]
	Folate	Rifampicin	Tuberculosis	Date et al. [226]
	Folate	Vancomycin	*Staphylococcus aureus*	Chakraborty et al. [227]
	TAT (trans-activating transcriptor) peptide	Ritonavir	HIV	Rao et al. [228]
	Transferrin anchored pegylated albumin nanoparticles (Tf-PEG-NPs)	Azidothymidine	HIV	Mishra et al. [229]
	Transferrin	Saquinavir	HIV	Ulbrich et al. [230]
	Mannan	Diadanosine	HIV	Kaur et al. [231]
SLN	Mannan	Gene delivery	Alveolar macrophages	Yu et al. [232]
	Mannose	Rifabutin	Alveolar macrophages	Nimje et al. [233]
	Transferrin	Quinine HCl	Malaria	Gupta et al. [234]
Dendrimers	Mannose	-	Macrophages	Gao et al. [235]
	Tuftsin	Efavirenz (EFV)	HIV	Dutta et al. [236, 237]
	Mannose	Rifampicin	Tuberculosis	Kumar et al. [238]
Carbon Nanotubes	Mannose	Amphotericin B	Macrophages	Pruthi et al. [239]

3.9 Specialised Targeting Approaches for Important Infectious Diseases

3.9.1 Tuberculosis

Tuberculosis is persistent and deadly infectious disease, caused *Mycobacterium tuberculosis* which is non-specifically phagocytosed by alveolar macrophages. The emergence of various resistance forms of tuberculosis has accelerated research in specific approaches to target the *M. Tuberculosis*. Date et al. developed folate

anchored polymeric nanoparticles of rifampicin and demonstrated 480 % enhancement in rifampicin uptake as compared to 300 % in the absence of folate in the human macrophage cell line U-937 [226]. Folate receptors enable flotillin-1 and caveolin receptor mediated endocytosis, thereby bypassing normal phagolysome formation to deliver the nanocarriers into the cytoplasm [191]. Lemmer et al. developed mycolic acid (MA) anchored nanoparticles (NP) of isoniazid. MA nanoparticles exhibited macrophage uptake, possibly localising in the cytoplasm. Verma et al. developed inhalable microparticles containing NO donors for the treatment of *Mycobacterium tuberculosis*. Such inhalable microparticles specifically delivered NO donors inside macrophages and showed sustain release in the cytosol. The antimycobacterial activity of microparticles was confirmed by the decrease in the *M. tuberculosis* CFU by up to 3-log in 24 h. The activity could be attributed to interaction of NO with bacterial DNA, lipids and protein. This strategy could be considered practical as the doses of NO donors (isosorbide nitrate) were much lower than those required for cardiovascular effects [240].

3.9.2 Malaria

Malaria is a complex disease caused by plasmodium and majorly resides in non-RES cells like red blood cells (RBCs) and hepatocytes. Entry of the parasite into the brain causes cerebral malaria. Malaria can be targeted at the exoerythrocytic stage by targeting RBCs, or targeting the hypnozoites to tackle malarial relapse and further in case of cerebral malaria targeting the brain. Increased permeability of infected RBCs is seen after 12–16 h of plasmodium invasion through formation of channels. These channels are "new permeability pathways" (NPPs) which allow entry of molecules such as dextran, protein A and IgG2a antibody thereby differentiating the non-infected and infected RBCs. Such pathways could be targeted to enable high drug loading in the erythrocytes specifically through design of nanocarriers of <80 nm [241]. This could be supported through design of stealth nanocarriers which could enable long circulation, using various stealth agents like poly(ethyleneglycol) (PEG), Pluronic, etc. [242]. Chloroquine liposomes anchored with anti-erythrocyte F (ab′)2 were studied for targeting to erythrocytes [243]. Hepatocytes the residence of hypnozoites expresses the asialoglycoprotein receptor (ASGPR), which is overexpressed in infections. Targeting this receptor using nanocarriers anchored with ASGPR ligands is a strategy for hepatocyte targeting. Joshi et al. prepared in situ primaquine nanocarboplex of primaquine phosphate anchored with pullulan as the ASGPR ligand for specific targeting to hepatocytes. Significantly, enhanced hepatic accumulation with preferential accumulation in the hepatocytes and a high hepatocytes/nonparenchymal cells ratio of 75:25 confirmed hepatocyte targeting [244]. Transferrin (Tf)-anchored solid lipid nanoparticles (SLNs) were intravenously administered for targeting quinine dihydrochloride to the brain, in cerebral malaria. Compared to conventional SLNs or drug solution the Tf-SLNs significantly enhanced the brain uptake of quinine [234].

3.9.3 HIV

A major feature of HIV that complicates therapy is the existence of HIV in multiple reservoirs, which include various cellular and anatomical sites [245]. The typical reservoirs are the liver, spleen, lungs, GIT and genital tract with the brain and bone marrow representing remote sites [246]. Targeted delivery for HIV therefore needs to address delivery to maximum sites simultaneously to achieve remission. One strategy that we propose is a combination of nanocarriers of size <100 nm to target remote sites and size >200 nm target major RES organs (Unpublished data). Viral replication is inhibited by the antioxidant glutathione. Erythrocytes containing glutathione (GSH) in combination with azidothymidine (AZT) and didanosine (DDI) showed higher reduction in viral DNA in bone marrow and brain as compared to DDI+GSH alone [247]. Immunoliposomes containing siRNA for targeting the lymphocyte function-associated antigen-1 (LFA-1) integrin, which is expressed on all leukocytes, was selectively taken up by T cells and macrophages, the primary site of HIV. Further, in vivo administration of anti-CCR5 siRNA resulted in leukocyte-specific gene silencing that was sustained for 10 days [248]. Nanogels comprising non-reverse transcriptase inhibitors (NRITs) decorated with a peptide for brain specific apolipoprotein E (apoE) receptors, showed tenfold suppression of retroviral activity and decrease inflammation in humanised mouse model of HIV-1 infection in the brain [249].

3.10 Veterinary Applications of Targeted Drug Delivery Systems

Targeted drug delivery for the therapy of veterinary infections assumes immense importance not only for improved animal health but due to the challenges posed by zoonotic diseases. About 13 zoonotic diseases including brucellosis, tuberculosis, trypanosomiasis, cysticercosis and others are related to 2.4 billion cases of infection in humans and over two million deaths annually [166, 167]. Such infections exist both in domestic animals and wild life. The close proximity of humans especially with such domestic animals is a cause of global concern. The WHO policy of "Cull and Kill" results not only in the loss of lives but also heavy monetary losses to the farmer. Targeted treatment strategies using nanodrug delivery systems could provide a revolutionary strategy to benefit both the animals and man. The benefits of targeted nanomedicine strategies are slowly gaining recognition as evident from a number of reported studies. Liposomes have been used by many researchers for treating various veterinary diseases such as Leishmaniasis [250, 251], Brucellosis [252], Blastomycosis [253], Babesiosis [254], etc. Patil et al. [171] developed an asymmetric lipomer. This is a combination of polymer–lipid containing doxycycline which could have application in the treatment of intracellular infections that are primarily resident in the spleen like brucellosis, ehrlichiosis, etc. A number of studies are reported on horses infected with babesiosis, *Streptococcus equi, T. gondii*

and *Strongylus vulgaris* infections using liposomes [254], polymeric nanospheres [255], dendrimers [256] and micelles [257] respectively. A recent study revealed the improved therapy of theileiriosis in cattle with solid lipid nanoparticles (SLN) of buparvaquone [258]. SLN revealed comparable effect with the intramuscular injection at significantly lower doses. Nanodrug delivery systems have also been evaluated in dogs, sheep and pigs. For details on nanodrug delivery applications in targeted delivery in veterinary infections, readers are directed to the following reference [259].

3.11 Future Scope

Targeted delivery for infectious diseases has immense scope. Tackling infections using nanodrug delivery systems could provide a practical alternative as a short term strategy. A rate-limiting factor however would be the serious concerns of toxicity. Nanodrug delivery systems due to their high intracellular delivery could precipitate new and unknown toxicities. Evolving strategies to predict the same is an important path forward. While vaccines could probably provide the ultimate cure and control, vaccine development is a complex process and not yet easily attained as evident from the limited success stories. However, designing nano-vaccines targeted to exhibit greater cellular response is also a near future prospect.

References

1. Terry W, Clos D (2013) Pentraxins: structure, function, and role in inflammation. ISRN inflamm 1:101
2. Hoppe HJ, Reid KB (1994) Collectins – soluble proteins containing collagenous regions and lectin domains – and their roles in innate immunity. Protein Sci 3:1143–1
3. Ren Y, Ding Q, Zhang X (2014) Ficolins and infectious diseases. Virol Sin 29(1):25–32
4. Poltorak A et al (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085–2088
5. Hoshino K et al (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 162:3749–3752
6. Qureshi ST et al (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med 189:615–625
7. Alexopoulou L, Medzhitov HA, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732–738
8. Takeuchi O et al (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443–451
9. Hemmi H et al (2000) A Toll-like receptor recognizes bacterial DNA. Nature 408:740–745
10. Hayashi F et al (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor. Nature 410:1099–1103
11. Rabinovitch M (1995) Professional and non-professional phagocytes: an introduction. Trends Cell Biol 5:85–87
12. Krombach F et al (1997) Cell size of alveolar macrophages: an interspecies comparison. Environ Health Perspect 105(5):1261–1263

13. Lewis CE, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66:605
14. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958–969
15. Swanson MS, Fernandez-Moreira E (2002) A microbial strategy to multiply in macrophages: the pregnant pause. Traffic 3(3):170–17
16. Vergne I, Chua J, Lee HH, Lucas M, Belisle J, Deretic V (2005) Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 102(11):4033–4038
17. Pujol C, Klein KA, Romanov GA, Palmer LE, Cirota C, Zhao Z, Bliska JB (2009) Yersinia pestis can reside in autophagosomes and avoid xenophagy in murine macrophages by preventing vacuole acidification. Infect Immun 77(6):2251–2261
18. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG (1994) Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263:678–681
19. Frehel C, Rastogi N (1987) Mycobacterium leprae surface components intervene in the early phagosome-lysosome fusion inhibition event. Infect Immun 55(12):2916–2921
20. Alvarez DC, Roberts R, Stahl PD (1997) Internalized Listeria monocytogenes modulates intracellular trafficking and delays maturation of the phagosome. J Cell Sci 6:731–743
21. Buchmeier NA, Heffron F (1991) Inhibition of macrophage phagosome-lysosome fusion by Salmonella typhimurium. Infect Immun 59(7):2232–2238
22. Desjardins M, Descoteaux A (1997) Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. J Exp Med 185(12):2061–2068
23. Mosser DM, Miles SA (2007) Avoidance of innate immune mechanisms by the protozoan parasite, Leishmania spp in protozoans in macrophages. Landes Biosci 9:118–124
24. Sibley LD, Weidner E, Krahenbuhl JL (1985) Phagosome acidification blocked by intracellular Toxoplasma gondii. Nature 315(6018):416–419
25. Borlace GN, Jones HF, Keep SJ, Butle R, Brooks DA (2011) Helicobacter pylori phagosome maturation in primary human macrophages. Gut Pathog 3:3
26. Ochatt CM, Ullo M et al (1993) Characterization of the catalytic subunit of Trypanosoma cruzi cyclic AMP-dependent protein kinase. Mol Biochem Parasitol 57(1):73–81
27. Roy CR (2005) Trimming the fat: a Brucella abortus survival strategy. Nat Immunol 6(6):546
28. Ghosh M, Bandyopadhyay S (2004) Interaction of Leishmania parasites with dendritic cells and its functional consequences. Immunobiology 209(1–2):173–177
29. Swanson MS, Isberg RR (1995) Association of Legionella pneumophila with the macrophage endoplasmic reticulum. Infect Immun 63(9):3609–3620
30. Berger KH, Isberg RR (1993) Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila. Mol Microbiol 7(1):7–19
31. Ray K, Marteyn B, Sansonetti PJ, Tang CM (2009) Life on the inside: the intracellular lifestyle of cytosolic bacteria. Nat Rev Microbiol 7(5):333–340
32. Dermine JF, Desjardins M (1999) Survival of intracellular pathogens within macrophages. Protoplasma 210(1–2):11–24
33. Dramsi S, Cossart P (2002) Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. J Cell Biol 156(6):943–946
34. Whitworth T, Popov VL, Yu XJ, Walker DH, Bouyer DH (2005) Expression of the Rickettsia prowazekii pld or tlyC gene in Salmonella enterica serovar typhimurium mediates phagosomal escape. Infect Immun 73:6668–6673
35. Mumy KL, Bien JD, Pazos MA, Gronert K, Hurley BP, McCormick BA (2008) Distinct isoforms of phospholipase A2 mediate the ability of Salmonella enterica serotype typhimurium and Shigella flexneri to induce the transepithelial migration of neutrophils. Infect Immun 76(8):3614–3627
36. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, Golda A, Maciag-Gudowska A, Brix K, Shaw L, Foster T, Potempa J (2008) A potential new pathway for Staphylococcus aureus dissemination: the silent survival of S. aureus phagocytosed by human monocyte-derived macrophages. PLoS One 3(1):1409

37. Shin JS, Gao Z, Abraham SN (2000) Involvement of cellular caveolae in bacterial entry into mast cells. Science 289:785–788
38. Sibley LD, Charron AJ, Hakansson S, Mordue DG (2007) Invasion and intracellular survival by Toxoplasma in Protozoans in Macrophages. Landes Bioscience 16–21
39. Dabiri GA, Sanger JM, Portnoy DA, Southwick FS (1990) Listeria monocytogenes moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc Natl Acad Sci U S A 87(16):6068–6072
40. Van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, Brenner M, Peters PJ (2007) M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell 129:1287–1298
41. Schroeder GN, Hilbi H (2008) Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin Microbiol Rev 21(1):134–156
42. Santic M, Asare R, Skrobonja I, Jones S, Abu KY (2008) Acquisition of the vacuolar ATPase proton pump and phagosome acidification are essential for escape of Francisella tularensis into the macrophage cytosol. Infect Immun 76(6):2671–2677
43. Andrews NW (1994) From lysosomes into the cytosol: the intracellular pathway of Trypanosoma cruzi. Braz J Med Biol Res 27(2):471–475
44. Winkler HH, Daugherty RM (1983) Cytoplasmic distinction of avirulent and virulent Rickettsia prowazekii: fusion of infected fibroblasts with macrophage-like cells. Infect Immun 40(3):1245–1247
45. Alexander J, Vickerman K (1975) Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of Leishmania mexicana-infected macrophages. J Protozool 22:502–508
46. Clemens DL, Horwitz MA (1993) Hypoexpression of major histocompatibility complex molecules on Legionella pneumophila phagosomes and phagolysosomes. Infect Immun 61(7):2803–2812
47. Burton PR, Stueckemann J, Welsh RM, Paretsky D (1978) Some ultrastructural effects of persistent infections by the rickettsia Coxiella burnetii in mouse L cells and green monkey kidney (Vero) cells. Infect Immun 21:556–566
48. Straley SC, Harmon PA (1984) Yersinia pestis grows within phagolysosomes in mouse peritoneal macrophages. Infect Immun 45(3):655–659
49. Miller M, Dreisbach A, Otto A, Becher D, Bernhardt J, Hecker M, Peppelenbosch MP, van Dijl JM (2011) Mapping of interactions between human macrophages and Staphylococcus aureus reveals an involvement of MAP kinase signaling in the host defense. J Proteome Res 10(9):4018–4032
50. Gatfield J et al (2000) Essential role for cholesterol in entry of mycobacteria into macrophages. Science 288:1647–1650
51. Catron DM, Sylvester MD, Lange Y, Kadekoppala M, Jones BD, Monack DM, Falkow S, Haldar K (2002) The Salmonella containing vacuole is a major site of intracellular cholesterol accumulation and recruits the GPI-anchored protein CD55. Cell Microbiol 4:315–328
52. Simons K, Ehehalt R (2002) Cholesterol, lipid rafts, and disease. J Clin Invest 110(5):597–603
53. Miller BH, Fratti RA, Poschet JF, Timmins GS, Master SS, Burgos MM, Marletta MA, Deretic V (2004) Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. Infect Immun 72(5):2872–2878
54. Schlesinger LS (1996) Entry of Mycobacterium tuberculosis into mononuclear phagocytes. Curr Top Microbiol Immunol 215:71–96
55. Brown EJ (1991) Complement receptors and phagocytosis. Curr Opin Immunol 3:76–82
56. Schorey JS et al (1997) A macrophage invasion mechanism of pathogenic mycobacteria. Science 277:1091–1093
57. Schlesinger LS (1993) Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. J Immunol 150(7):2920–2930
58. Armstrong JA, Hart PDA (1971) Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J Exp Med 134:713–740

59. Zimmerli S et al (1996) Selective receptor blockade during phagocytosis does not alter the survival and growth of Mycobacterium tuberculosis in human macrophages. Am J Respir Cell Mol Biol 15:760–770
60. Ernst JD (1998) Macrophage receptors for Mycobacterium tuberculosis. Infect Immun 66:1277–1281
61. Romling U, Sierralta WD, Eriksson K, Normark S (1998) Multicellular and aggregative behavior of Salmonella typhimurium strains is controlled by mutations in the agfD promoter. Mol Microbiol 28:249–264
62. Anriany YA, Weiner RM, Johnson JA, Rezende CA, Joseph SW (2001) Salmonella enterica serovar Typhimurium DT104 displays a rugose phenotype. Appl Environ Microbiol 67:4048–4056
63. Petter G, Keller J, Rahman LH, Carlson MM, Silvers S (1996) A novel relationship between O-antigen variation, matrix formation, and invasiveness of Salmonella enteritidis. Epidemiol Infect 117:219–231
64. White AP, Gibson DL, Kim W, Kay WW, Surette MG (2006) Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella. J Bacteriol 188:3219–3227
65. Collinson SK et al (1993) Thin, aggregative fimbriae mediate binding of Salmonella enteritidis to fibronectin. J Bacteriol 175:12–18
66. Zogaj XM, Nimtz M, Rohde M, Bokranz W, Romling U (2001) The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39:1452–1463
67. Ryu JH, Beuchat LR (2005) Biofilm formation by Escherichia coli O157:H7 on stainless steel: effect of exopolysaccharide and Curli production on its resistance to chlorine. Appl Environ Microbiol 71:247–254
68. Scher K, Romping U, Yaron S (2005) Effect of heat, acidification, and chlorination on Salmonella enterica serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 71:1163–1168
69. Solano CB, Garcia JV, Berasain C, Ghigo GM, Gamazo C, Lasa I (2002) Genetic analysis of Salmonella enteritidis biofilm formation: critical role of cellulose. Mol Microbiol 43:793–808
70. Gibson DL, White AP, Snyder SD, Martin S (2006) Salmonella produces an O-antigen capsule regulated by AgfD and important for environmental persistence. J Bacteriol 188(22):7722
71. Teixeir HD, Schumacher RI, Meneghini R (1998) Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase. Proc Natl Acad Sci U S A 95:7872–7875
72. Fries BC, Taborda CP, Serfass E, Casadevall A (2001) Phenotypic switching of Cryptococcus neoformans occurs in vivo and influences the outcome of infection. J Clin Invest 108:1639–1648
73. Sebastiaan MB et al (2011) HIV-1 and the macrophages. Future Virol 6(2):187–208
74. Marechal V et al (2001) Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. J Virol 75(111):66–77
75. Gantt KR, Cherry S, Rodriguez N, Jeronimo SM, Nascimento ET et al (2003) Activation of TGF-beta by Leishmania chagasi: importance for parasite survival in macrophages. J Immunol 170:2613–2620
76. Bray RS, Heikal B, Kaye PM, Bray MA (1983) The effect of parasitization by Leishmania mexicana on macrophage function in vitro. Acta Trop 40:29–38
77. Olivier M, Gregory DJ, Forget G (2005) Subversion mechanisms by which immune response: a signaling point of leishmania parasites can escape the host. Clin Microbiol Rev 18(2):293
78. D'Orsogna MR, Mail TC (2009) Optimal cytoplasmic transport in viral infections. PLoS One 30(412):8165
79. Roop RM, Bellaire BH, Valderas MH, Cardelli JA (2004) Adaptation of the Brucellae to their intracellular niche. Mol Microbiol 52(3):621–630

80. Celli J, Chastellier CD, Franchini DM et al (2003) Brucella evades macrophage killing via virb-dependent sustained interactions with the endoplasmic reticulum. J Exp Med 198(4):545–556
81. Libraty DH et al (2002) Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis 85(9):1213–1221
82. Moradpoura D et al (2003) Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. Antivir Res 60(2):103–109
83. Heinz C, Falke D, Weise K, Bachmann M, Fonseca MC, Zaubitzer T, Müller WEG (1989) Change of processing and nucleocytoplasmic transport of mRNA in HSV-1-infected cells. Virus Res 13(1):61–78
84. Arnheiter H, Skuntz S, Noteborn M, Chang S, Meier E (1990) Transgenic mice with intracellular immunity to influenza virus. Cell 62(1):51–61
85. Tilney LG, Harb OS, Connelly PS, Robinson CG, Roy CR (2001) How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J Cell Sci 114(24):4637–4650
86. Handman E, Greenblatt CL, Goding JW (1984) An amphipathic sulphated glycoconjugate of Leishmania: characterization with monoclonal antibodies. EMBO J 3(10):2301–2306
87. Collins B et al (2012) Assessing the contributions of the LiaS histidine kinase to the innate resistance of listeria monocytogenes to nisin, cephalosporins, and disinfectants. Appl Environ Microbiol 78(8):2923–2929
88. Moulder J (1962) The biochemistry of intracellular parasitism. p 172
89. Trebichavsky I, Splichal I, Splichalova A (2010) Innate immune response in the gut against Salmonella - review. Folia Microbiol (Praha) 55(3):295–300
90. Skvortsov TA, Azhikina TL (2012) Adaptive changes in gene expression of Mycobacterium tuberculosis during the development of the infection. Russ J Bioorg Chem 38(4):341–353
91. Khodor S, Kwaik YA (2010) Triggering Ras signalling by intracellular Francisella tularensis through recruitment of PKCa and bI to the SOS2/GrB2 complex is essential for bacterial proliferation in the cytosol. Cell Microbiol 12(11):1604–1621
92. Herberman RB, Ortaldo JR (1981) Natural killer cells: their role in defenses against disease. Science 214(2):24
93. Ojo E, Wigzell H (1978) Natural killer cells may be the only cells in normal mouse lymphoid cell populations endowed with cytolytic ability for antibody-coated tumour target cells. Scand J Immunol 7:297
94. Lopez C (1980) Genetic control of natural resistance to infection and malignancy. p 253
95. Bancroft GJ, Shellam GR, Chalmer JE (1981) Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. J Immunol 126:988
96. Welsh RM (1981) Natural cell-mediated immunity during viral infections. Curr Top Microbiol Immunol 92:83–106
97. Golub ES (1980) The cellular basis of the immune response. an approach to immunobiology. Sinauer, Sunderland, MA
98. Powrie F, Maloy KJ (2003) Regulating the regulators. Immunology 299(5609):1030–1031
99. Mills KH (2004) Regulatory T cells: friend or foe in immunity to infection? Nat Rev Immunol 4:841–855
100. Belkaid Y, Rouse BT (2005) Natural regulatory T cells in infectious disease. Nat Immunol 6:353–360
101. N'Diaye EN, Darzacq X, Astarie-Dequeker C, Daffe M, Calafat J, Maridonneau- Parini I (1998) Fusion of azurophil granules with phagosomes and activation of the tyrosin ekinase Hck are specifically inhibited during phagocytosis of mycobacteria by human neutrophils. J Immunol 161:4983–4991
102. Roy CR, Berger KH, Isberg RR (1998) Legionella pneumophila DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol Microbiol 28:663–674

103. Gao LY, Abu KY (1999) Apoptosis in macrophages and alveolar epithelial cells during early stages of infection by Legionella pneumophila and its role in cytopathogenicity. Infect Immun 67:862–870
104. Segal G, Shuman HA (1999) Legionella pneumophila utilizes the same genes to multiply within Acanthamoeba castellanii and human macrophages. Infect Immun 67:2117–2124
105. Gomes MS, Paul S, Moreira AL, Appelberg R, Rabinovitch M, Kaplan G (1999) Survival of Mycobacterium avium and Mycobacterium tuberculosis in acidified vacuoles of murine macrophages. Infect Immun 67:3199–3206
106. Liautard JP, Gross A, Dornand J, Kohler S (1996) Interactions between professional phagocytes and Brucella spp. Microbiologia 12:197–206
107. Cole SP, Eckmann L, Guiney DG (1998) Nitric oxide-mediated crosstalk between Helicobacter and gastric mucosa. In: 38th interscience conference of antimicrobial agents and chemotherapy. 24–27 Sept, San Diego, CA. Abstract B15
108. Costerton JW, Stewart PS, Greenberg ES (1999) Bacterial biofilms: a common cause of persistent infections. Science 284(5418):1318–1322
109. Brown MR, Allison DG, Gilbert P (1988) Growth rate control of adherent bacterial populations. J Antimicrob Chemother 22:777
110. Cheema MS, Rassing JE, Marriott C (1986) The diffusion characteristics of antibiotics in mucus glycoprotein gels. J Pharm Pharmacol 38(S12):53
111. Stewart PS (1998) A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotechnol Bioeng 59:261
112. Piddock LJV (2006) Multidrug-resistance efflux pumps not just for resistance. Nat Rev Immunol 4(8):629–639
113. Paulsen IT (2003) Multidrug efflux pumps and resistance: regulation and evolution. Curr Opin Microbiol 6:446–451
114. Nikaido H, Zgurskaya HI (1999) Antibiotic efflux mechanisms. Curr Opin Infect Dis 12(6):529–536
115. Neyfakh AA (1992) The multidrug efflux transporter of Bacillus subtilis is a structural and functional homolog of the Staphylococcus NorA protein. Antimicrob Agents Chemother 36:484–485
116. Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M (1990) Nucleotide sequence and characterization of the Staphylococcus aureus nor A gene, which confers resistance to quinolones. J Bacteriol 172:6942–6949
117. Kaatz GW, Seo SM, Ruble CA (1993) Efflux-mediated fluoroquinolone resistance in Staphylococcus aureus. Antimicrob Agents Chemother 37:1086–1094
118. Wright GD (2005) Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv Drug Deliv Rev 57:1451–1470
119. Torchillin VP (2000) Drug targeting. Eur J Pharmaceut Sci 11(2):S81–S91
120. Garnett MC (2001) Targeted drug conjugates: principles and progress. Adv Drug Deliv Rev 53(2):171–216
121. Vasir Jaspreet K, Reddy MK, Labhasetwar VD (2005) Nanosystems in drug targeting, opportunities and challenges. Curr Nanosci 1:47–64
122. Schiffelers RM et al. (2001) Targeted drug delivery to enhance efficacy and shorten treatment duration in disseminated Mycobacterium avium infection in mi host factors influencing the preferential localization of sterically stabilized liposomes in klebsiella pneumoniae-infected rat lung tissue. IAJM Pharm Res 18:780–787
123. Edens HA, Levi BP, Jaye DL, Walsh S, Reaves TA, Turner JR, Nusrat A, Parkos CA (2002) Neutrophil transepithelial migration: evidence for sequential, contact-dependent signaling events and enhanced paracellular permeability independent of transjunctional migration. J Immunol 169:476–486
124. Maeda H et al (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 65(1–2):271–284
125. Powers KW, Palazuelos M, Moudgil BM, Roberts SM (2007) Characterization of the size, shape, and state of dispersion of nanoparticles for toxicological studies. Nanotoxicology 1:42

126. Duan X, Li Y (2013) Physicochemical characteristics of nanoparticles affect. Small 9(9–10):1521–1532
127. Gratton SEA, Ropp PA, Pohlhaus PD et al (2008) The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci U S A 105:11613
128. Hillaireau HP, Couvreur C (2009) Nanocarriers entry into the cell: relevance to drug delivery. Cell Mol Life Sci 66:2873
129. Alipoura M, Halwania M, Omria A et al (2008) Antimicrobial effectiveness of liposomal polymyxin B against resistant Gram-negative bacterial strains. Int J Pharm 355(1–2):293–298
130. Mehta RT et al (1993) In vitro activities of free and liposomal drugs against Mycobacterium avium-M. intracellular complex and M. tuberculosis. Antimicrob Agents Chemother 37(12):2584–2587
131. El-Ridy MS, Mostafa DM, Shehab BA, Nasr ES, Abd El-Alim S (2007) Biological evaluation of pyrazinamide liposomes for treatment of Mycobacterium tuberculosis. Int J Pharm 330(1–2):82–88
132. Gaspara MM, Cruz A et al (2008) Rifabutin encapsulated in liposomes exhibits increased therapeutic activity in a model of disseminated tuberculosis. Int J Antimicrob Agents 31(1):37–45
133. Fattal E et al (1991) Liposome-entrapped ampicillin in the treatment of experimental murine listeriosis and salmonellosis. Antimicrob Agents Chemother 35(4):770
134. Fountain MW, Weiss SJ, Fountain AG (1985) Treatment of Brucella canis and Brucella abortus in vitro and in vivo by stable plurilamellar vesicle-encapsulated aminoglycosides. J Infect Dis 152(3):529–535
135. Magallanes M, Dijkstra J, Fierer J (1993) Liposome-incorporated ciprofloxacin in treatment of murine salmonellosis. Antimicrob Agents Chemother 37(11):2293–2297
136. Date AA, Joshi MD, Patravale VB (2007) Parasitic diseases: liposomes and polymeric nanoparticles versus lipid nanoparticles. Adv Drug Deliv Rev 59:505–521
137. Oussoren C et al (1999) Liposomes as carriers of the antiretroviral agent dideoxycytidine-5 %-triphosphate. Int J Pharm 180:261–270
138. Saraogia GK, Gupta P, Gupta UD, Jain NK et al (2010) Gelatin nanocarriers as potential vectors for effective management of tuberculosis. Int J Pharm 385:143–149
139. Kaur M, Malik B., Garg T, Rath G, Goyal AK (2014) Development and characterization of guar gum nanoparticles for oral immunization against tuberculosis. Drug Deliv. (Ahead of Print): 1–7
140. Booysen LL et al (2013) In vivo/in vitro pharmacokinetic and pharmacodynamic study of spray-dried poly-(dl-lactic-co-glycolic) acid nanoparticles encapsulating rifampicin and isoniazid. Int J Pharm 444(28):10–17
141. Esmaeili F et al (2007) Preparation and antibacterial activity evaluation of rifampicin-loaded poly lactide-co-glycolide nanoparticles. Nanomedicine 3(2):161–167
142. Dou H et al (2009) Macrophage delivery of nanoformulated antiretroviral drug to the brain in a murine model of NeuroAIDS. J Immunol 183(1):661–669
143. Toti US et al (2011) Targeted delivery of antibiotics to intracellular chlamydial infections using PLGA nanoparticles. Biomaterials 32:6606–6613
144. Tyagi R et al (2005) Targeted delivery of arjunglucoside I using surface hydrophilic and hydrophobic nanocarriers to combat experimental leishmaniasis. J Drug Target 13(3):161–171
145. Zhang Q, Liao G, Wei D, Nagai T (1998) Increase in gentamicin uptake by cultured mouse peritoneal macrophages and rat hepatocytes by its binding to polybutylcyanoacrylate nanoparticles. Int J Pharm 164:21–27
146. Azarmi S, Roa WH, Löbenberg R (2008) Targeted delivery of nanoparticles for the treatment of lung diseases. Adv Drug Deliv Rev 60(8):863–875
147. Kisich KO et al (2007) Encapsulation of moxifloxacin within poly(butyl cyanoacrylate) nanoparticles enhances efficacy against intracellular Mycobacterium tuberculosis. Int J Pharm 345(1–2):154–162

148. Haas SE et al (2009) Nanoencapsulation increases quinine antimalarial efficacy against Plasmodium berghei in vivo. Int J Antimicrob Agents 34:156–161
149. Espuelas MS, Legrand P, Loiseau PM et al (2002) In vitro antileishmanial activity of amphotericin B loaded in poly(ε-caprolactone) nanospheres. J Drug Target 10(8):593–599
150. Yadav AB et al (2009) Inhalable microparticles containing isoniazid and rifabutin target macrophages and "stimulate the phagocyte" to achieve high efficacy. Indian J Exp Biol 47(6):469–474
151. Zhoua H, Zhang Y, Biggs DL, Mark C et al (2005) Microparticle-based lung delivery of INH decreases INH metabolism and targets alveolar macrophages. J Control Release 107(2):288–299
152. Alex MR, Chacko AJ, Jose S, Souto EB (2011) Lopinavir loaded solid lipid nanoparticles (SLN) for intestinal lymphatic targeting. Eur J Pharm Sci 42:11–18
153. Bargoni A et al (2001) Transmucosal transport of tobramycin incorporated in solid lipid nanoparticles after duodenal administration to rats. Part ii tissue distribution. Pharmacol Res 43(5):497
154. Heiatia H, Rashad T, Richard R, Phillips NC (1997) Solid lipid nanoparticles as drug carriers Incorporation and retention of the lipophilic prodrug 3´-azido-3´-deoxythymidine palmitate. Int J Pharm 146(1):123–131
155. Chattopadhyay N, Jason ZW et al (2008) Solid lipid nanoparticles enhance the delivery of the HIV protease inhibitor, atazanavir, by a human brain endothelial cell line. Pharm Res 25:10
156. Bhandari R, Kaur IP (2013) Pharmacokinetics, tissue distribution and relative bioavailability of isoniazid-solid lipid nanoparticles. Int J Pharm 441(1–2):202–212
157. Clemens DL et al (2012) Targeted intracellular delivery of antituberculosis drugs to mycobacterium tuberculosis-infected macrophages via functionalized mesoporous silica nanoparticles. Antimicrob Agents Chemother 56(5):2535–2545
158. Singh G, Dwivedi H, Saraf SK, Saraf SA (2011) Niosomal delivery of isoniazid development and characterization. Trop J Pharm Res 10(2):203–210
159. Singh KK, Vingkar SK (2008) Formulation, antimalarial activity and biodistribution of oral lipid nanoemulsion of primaquine. Int J Pharm 347(1–2):136–143
160. Falk R, Domb AJ, Polacheck I (1999) A novel injectable water-soluble amphotericin B-arabinogalactan conjugate. Antimicrob Agents Chemother 43(8):1975–1981
161. Dutta T, Jain NK (2007) Targeting potential and anti-HIV activity of lamivudine loaded mannosylated poly (propyleneimine) dendrimer. Biochim Biophys Acta 1770(4):681–686
162. Bhadra D, Yadav AK, Bhadra S, Jain NK (2005) Glycodendrimeric nanoparticulate carriers of primaquine phosphate for liver targeting. Int J Pharm 295:221–233
163. Prajapati VK et al (2011) Targeted killing of Leishmania donovani in vivo and in vitro with amphotericin B attached to functionalized carbon nanotubes. J Antimicrob Chemother 66(4):874–879
164. Ren G, Dawei H, Cheng E, Vargas-Reus MA, Reip P, Allaker AP (2009) Characterisation of copper oxide nanoparticles for antimicrobial applications. Int J Antimicrob Agents 33(6):587–590
165. Raffi M, Saba M (2010) Investigations into the antibacterial behavior of copper nanoparticles against Escherichia coli. Ann Microbiol 60(1):75–80
166. Jones KE, Patel NG, Levy MA. Storeygard A, Balk D, Gittleman JL, Daszak P (2008) Global trends in emerging infectious diseases. Nature. 451: 990–993
167. Jones N et al (2008) Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS Microbiol Lett 279(1):71
168. Chatterjee S, Bandyopadhyay A, Keka S (2011) Effect of iron oxide and gold nanoparticles on bacterial growth leading towards biological application. J Nanobiotechnology 9(34):1
169. Champion JA, Mitragotri S (2006) Role of target geometry in phagocytosis. PNAS 103(13):4930–4934
170. Sharma G et al (2010) Polymer particle shape independently influences binding and internalization by macrophages. J Control Release 147:408
171. Patil RR, Gaikwad RV, Samad A, Devarajan PV (2008) Role of lipids in enhancing splenic uptake of polymer-lipid (LIPOMER) nanoparticles. J Biomed Nanotechnol 4(3):359–366

172. Devarajan PV, Jindal AB, Patil R et al (2010) Particle shape: a new design parameter for passive targeting in splenotropic drug delivery. J Pharm Sci 99(6):2576
173. Schipper ML et al (2009) Particle size, surface coating, and PEGylation influence the biodistribution of quantum dots in living mice. Small 5:126
174. Esmaeili F, Ghahremani F, Esmaeili B, Khoshayand MR, Atyabi F, Dinarvand R (2008) PLGA nanoparticles of different surface properties: preparation and evaluation of their body distribution. Int J Pharm 349:249
175. Verma A, Stellacci F (2010) Effect of surface properties on nanoparticle–cell interactions. Small 6(1):12–21
176. Roser M, Fischer D, Kissel T (1998) Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats. Eur J Pharm Biopharm 46:255
177. Blau S, Jubeh TT, Haupt SM, Rubinstein A (2000) Drug targeting by surface cationization. Crit Rev Ther Drug Carrier Syst 17(5):425
178. Xiao K, Li Y, Luo J, Lee JS, Xiao W, Gonik AM, Agarwal RG, Lam KS (2011) The effect of surface charge on in vivo biodistribution of PEG-oligocholic acid based micellar nanoparticles. Biomaterials 32:3435
179. Rigotti A, Susan AL, Krieger M (1995) The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. J Biol Chem 270:16221–16224
180. Ahsana F et al (2002) Targeting to macrophages: role of physicochemical properties of particulate carriers liposomes and microspheres on the phagocytosis by macrophages. J Control Release 79(1–3):19
181. Nishioka Y, Yoshino H (2001) Lymphatic targeting with nanoparticulate system. Adv Drug Deliv Rev 479(1):55–64
182. Bareford LM, Swaan PW (2007) Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev 59(8):748–758
183. Albanese A, Peter ST, Chan CW (2012) The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu Rev Biomed Eng 14:1–16
184. Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. Annu Rev Biochem 78:857–902
185. Iversena T, Sandvig K (2011) Endocytosis and intracellular transport of nanoparticles: present knowledge and need for future studies. Nano Today 6(2):176–185
186. Ford MG et al (2001) Simultaneous binding of PtdIns P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science 291(105):1–55
187. Roth TF, Porter KR (1964) Yolk protein uptake in the oocyte of the mosquito Aedes aegypti. L. J Cell Biol 20:313–332
188. Parton RG, Simons K (2007) The multiple faces of caveolae. Nat Rev Mol Cell Biol 8:185–194
189. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG (1992) Caveolin, a protein component of caveolae membrane coats. Cell 68:673–682
190. Krajewska WM, Maslowska I (2004) Caveolins: structure and function in signal transduction. Cell Mol Biol Lett 9:195–220
191. Glebov OO, Bright NA, Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol 8:46–54
192. Frick M, Bright NA, Riento K, Bray A, Merrified C, Nichols BJ (2007) Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Biol 17:1151–1156
193. Lundmark R, Doherty GJ, Vallis Y, Peter BJ, McMahon HT (2008) Arf family GTP loading is activated by, and generates, positive membrane curvature. Biochem J 414(2):189–194
194. Naslavsky N, Weigert R, Donaldson JG (2004) Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. Mol Biol Cell 15:3542–3552
195. Donaldson JG, Porat S, Cohen LA (2009) Clathrin-independent endocytosis: a unique platform for cell signaling and PM remodelling. Cell Signal 21:1–6
196. Kirkham M, Parton RG (2005) Clathrin-independent endocytosis: new insights into caveolae and noncaveolar lipid raft carriers. Biochim Biophys Acta 1746:349–363

197. Marbet P, Rahner C, Stieger B, Landmann L (2006) Quantitative microscopy reveals 3D organization and kinetics of endocytosis in rat hepatocytes. Microsc Res Tech 69:693–707
198. Krueger EW, Orth JD, Cao H, McNiven MA (2003) A dynamin-cortactin-Arp2/3 complex mediates actin reorganization in growth factor-stimulated cells. Mol Biol Cell 14:1085–1096
199. Grassart A, Dujeancourt A, Lazarow PB, Dautry-Varsat A, Sauvonnet N (2008) Clathrin-independent endocytosis used by the IL-2 receptor is regulated by Rac1, Pak1 and Pak2. EMBO Rep 9:356–362
200. Lamaze C, Dujeancourt A, Baba T, Lo CG, Benmerah A, Dautry-Varsat A (2001) Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. Mol Cell 7:661–671
201. Ezekovvitz RA, Williamsi DJ, Kozieltil H et al (1991) Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. Nature 351(6322):155–158
202. Agrawal AK, Gupta CM (2000) Tuftsin-bearing liposomes in treatment of macrophage-based infections. Adv Drug Deliv Rev 41(2):135–146
203. Tzehoval E et al (1978) Tuftsin (an Ig-associated tetrapeptide) triggers the immunogenic function of macrophages: implications for activation of programmed cells. Proc Natl Acad Sci 75(7):3400
204. Wilkinson K, Khoury JEI (2012) Microglial scavenger receptors and their roles in the pathogenesis of Alzheimer's disease. Int J Alzheimers Dis 489456:1
205. Graversen JH, Svendsen P, Dagnæs-Hansen F, Dal J, Anton G, Etzerodt A, Petersen MD, Christensen PA, Møller HJ, Moestrup SK (2012) Targeting the hemoglobin scavenger receptor CD163 in macrophages highly increases the anti-inflammatory potency of dexamethasone. Mol Ther 20(8):1550–1558
206. Guilliams M, Pierre B, Van SY, Hamida H, Lambrecht BN (2014) The function of Fcγ receptors in dendritic cells and macrophages. Nat Rev Immunol 14:94–108
207. Taylor ME, Conary JT, Lennartz MR, Stahl PD, Drickamer K (1990) Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. J Biol Chem 265:12156–12162
208. Kroger PA, Sierra-Filardi E, Domínguez-Soto A, Samaniego R, Corcuera MT, Gomez-Aguado F, Ratnam M, Sánchez-Mateos P, Corbí AL (2009) Folate receptor β is expressed by tumor-associated macrophages and constitutes a marker for M2 anti inflammatory/regulatory macrophages. Cancer Res 69:9395
209. Heijden JWV, Oerlemans R, Ben AC, Dijkmans BA et al (2009) Folate receptor β as a potential delivery route for novel folate antagonists to macrophages in the synovial tissue of rheumatoid arthritis patients. Arthritis Rheum 60(1):12–21
210. Qian LH, Sun H, Ho K (2002) Targeted drug delivery via the transferrin receptor- mediated endocytosis pathway. Pharmacol Rev 54:561–587
211. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11:373–384
212. Campagne S, Wiesmann B (2007) Macrophage complement receptors and pathogen clearance. Cell Microbiol 9(9):2095–2102
213. Kotenko SV, Gallagher G, Baurin VV, Antes AL, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP (2002) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 4:69–77
214. Dobrovolskaia MA, Vogel S (2002) Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect 4(9):903–914
215. Banerjee G et al (1996) Drug delivery system: targeting of pentamidines to specific sites using sugar grafted liposomes. J Antimicrob Chemother 38(1):1145–1150
216. Chono S, Tanino T, Seki T, Morimoto K (2008) Efficient drug delivery to alveolar macrophages and lung epithelial lining fluid following pulmonary administration of liposomal ciprofloxacin in rats with pneumonia and estimation of its antibacterial effects. Drug Dev Ind Pharm 34(10):1090–1096
217. Galnoy YG, Zor T, Margalit R (2012) Hyaluronan-modified and regular multilamellar liposomes provide sub-cellular targeting to macrophages, without eliciting a pro-inflammatory response. J Control Release 1609(2):388–393

218. Kim J, Basak JM, Holtzman DM (2009) The role of apolipoprotein E in Alzheimer's disease. Neuron 63(3):287–303
219. Tempone AG et al (2004) Targeting Leishmania (L.) chagasi amastigotes through macrophage scavenger receptors: the use of drugs entrapped in liposomes containing phosphatidylserine. J Antimicrob Chemother 54(1):60–68
220. Deol P, Khuller GK, Joshi K (1997) Therapeutic efficacies of isoniazid and rifampin encapsulated in lung-specific stealth liposomes against Mycobacterium tuberculosis infection induced in mice. Antimicrob Agents Chemother 41(6):1211–1214
221. Vyas SP, Quraishi S, Gupta S, Jaganathan KS (2005) Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages. Int J Pharm 296(1–2):12–25
222. Derksen JTP, Morselt HWM, Scherphof GL (1988) Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages. Biochim Biophys Acta 971(2):127–136
223. Bestman-Smith J, Gourde P, Désormeaux A, Tremblay MJ, Bergeron MJ (2000) Sterically stabilized liposomes bearing anti-HLA-DR antibodies for targeting the primary cellular reservoirs of HIV-1. Biochim Biophys Acta 1468(1–2):161–174
224. Gagne JD et al (2002) Targeted delivery of indinavir to HIV-1 primary reservoirs with immunoliposomes. Biochim Biophys Acta 1558:198–210
225. Chaubey P et al (2014) Development and optimization of curcumin-loaded mannosylated chitosan nanoparticles using response surface methodology in the treatment of visceral leishmaniasis. Carbohydr Polym 101:1101–1108
226. Date PV, Patel MD, Majee SB, Abdul S, Devarajan PV (2013) Ionic complexation as a noncovalent approach for the design of folate anchored rifampicin gantrez nanoparticles. J Biomed Nanotechnol 9(5):765–775
227. Chattopadhyay S, Chakraborty SP, Laha D, Baral R, Pramanik P, Roy S (2012) Surface-modified cobalt oxide nanoparticles: new opportunities for anti-cancer drug development. Cancer Nanotechnol 3(1–6):13–23
228. Rao KS, Reddy S, Horning JL, Labhasetwar V (2008) TAT-conjugated nanoparticles for the CNS delivery of anti-HIV drugs. Biomaterials 29(33):4429–4438
229. Mishra V et al (2006) Targeted brain delivery of AZT via transferrin anchored pegylated albumin nanoparticles. J Drug Target 14(1):45–53
230. Ulbrich K et al (2009) Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood–brain barrier (BBB). Eur J Pharm Biopharm 71(2):251–256
231. Kaur A, Jain S, Tiwary A (2008) Mannan-coated gelatin nanoparticles for sustained and targeted delivery of didanosine: in vitro and in vivo evaluation. Acta Pharma 58(1):61–74
232. Yu W, Liu C, Liu Y, Zhang N, Xu W (2010) Mannan-modified solid lipid nanoparticles for targeted gene delivery to alveolar macrophages. Pharm Res 27(8):1584–1596
233. Nimje N et al (2009) Mannosylated nanoparticulate carriers of rifabutin for alveolar targeting. J Drug Target 17(10):777–787
234. Gupta Y, Jain A, Jain SK (2007) Transferrin-conjugated solid lipid nanoparticles for enhanced delivery of quinine dihydrochloride to the brain. J Pharm Pharmacol 59:935–940
235. Gao Q, Han J, Ma Z (2013) Polyamidoamine dendrimers-capped carbon dots/Au nanocrystal nanocomposites and its application for electrochemical immunosensor. Biosens Bioelectron 49:323–328
236. Dutta T et al (2008) Toxicological investigation of surface engineered fifth generation poly (propyleneimine) dendrimers in vivo. Nanotoxicology 2(2):62
237. Dutta T, Garg M, Jain NK (2008) Targeting of efavirenz loaded tuftsin conjugated poly (propyleneimine) dendrimers to HIV infected macrophages in vitro. Eur J Pharm Sci 3(4):181–189
238. Kumar VP, Asthana A, Dutta T, Jain NK (2006) Intracellular macrophage uptake of rifampicin loaded mannosylated dendrimers. J Drug Target 14(8):546–556
239. Pruthi J, Mehra NK, Jain NK (2012) Macrophages targeting of amphotericin B through mannosylated multiwalled carbon nanotubes. J Drug Target 20(7):593–604
240. Verma RK et al (2012) Inhalable microparticles containing nitric oxide donors: saying NO to intracellular mycobacterium tuberculosis. Mol Pharm 9:3183–3189

241. Charpian S, Przyborski JM (2008) Protein transport across the parasitophorous vacuole of Plasmodium falciparum: into the great wide open. Traffic 9:157–165
242. Vauthier C, Labarre D, Ponchel G (2007) Design aspects of poly(alkylcyanoacrylate) nanoparticles for drug delivery. J Drug Target 15(10):641–663
243. Agrawal AK, Singhal A, Gupta CM (1987) Functional drug targeting to erythrocytes in vivo using antibody bearing liposomes as drug vehicles. Biochem Biophys Res Commun 148:357–361
244. Joshi VM, Devarajan PV (2014) Receptor-mediated hepatocyte-targeted delivery of primaquine phosphate nanocarboplex using a carbohydrate ligand. Drug Deliv Transl Res 4:353–364
245. Blankson JN, Deborah P, Siliciano RF (2002) The challenge of viral reservoirs in hiv-1 infection. Annu Rev Med 53:557–593
246. Ramana KV (2012) HIV disease management in the highly active antiretroviral therapy (HAART) era. J Med Microbiol 1(1):101
247. Fraternale A, Casabianca A, Tonelli A, Chiarantini L, Brandi G, Magnani M (2001) New drug combinations for the treatment of murine AIDS and macrophage protection. Eur J Clin Investig 31(3):248–252
248. Kim SS et al (2010) RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice. Mol Ther 18(2):370–376
249. Gerson T, Makarov E, Senanayake TH, Santhi G, Larisa Y (2014) Nano-NRTIs demonstrate low neurotoxicity and high antiviral activity against HIV infection in the brain. Nanomedicine 10(1):177–185
250. Marques C, Carvalheiro M, Pereira MA, Jorge J, Cruz ME, Santos-Gomes GM (2008) Efficacy of the liposome trifluralin in the treatment of experimental canine leishmaniasis. Vet J 178:133–137
251. Ribeiro RR, Moura EP, Pimentel VM, Sampaio WM, Silva SM, Schettini DA (2008) Reduced tissue parasitic load and infectivity to sand flies in dogs naturally infected by Leishmania (Leishmania) chagasi following treatment with a liposome formulation of meglumine antimoniate. Antimicrob Agents Chemother 52:2564–2572
252. Nicoletti P, Lenk RP, Popescu MC, Swenson CE (1989) Efficacy of various treatment regimens, using liposomal streptomycin in cows with brucellosis. Am J Vet Res 50:1004–1007
253. Krawiec DR, McKiernan BC, Twardock AR, Swenson CE, Itkin RJ, Johnson LR, Kurowsky LK, Marks CA (1996) Use of an amphotericin B lipid complex for treatment of blastomycosis in dogs. J Am Vet Med Assoc 209:2073–2075
254. Timofeev BA, Bolotin IM, Stepanova LP, Bogdanov AA, Georgiu K, Malyshev SN, Petrovsky VV, Klibanov AL, Torchilin VP (1994) Liposomal diamidine (imidocarb), preparation and animal studies. J Microencapsul 11:627–632
255. Cubillos C et al (2008) Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. J Virol 82:7223–7230
256. Hiszczynska SE, Oledzka G, Gasior H, Li H, Xu JB, Sedcole R, Kur J, Bickerstaffe R, Stankiewicz M (2011) Evaluation of immune responses in sheep induced by DNA immunization with genes encoding GRA1, GRA4, GRA6 and GRA7 antigens of Toxoplasma gondii. Vet Parasitol 177:281–289
257. Klei TR, Torbert BJ, Chapman MR, Turk MA (1984) Efficacy of ivermectin in injectable and oral paste formulations against eight-week-old Strongylus vulgaris larvae in ponies. Am J Vet Res 45:183–185
258. Soni MK et al (2014) Buparvaquone loaded solid lipid nanoparticles for targeted delivery in theleriosis. J Pharm Bioall Sciences 6(1):22–30
259. Devarajan PV, Soni MKP (2014) Targeted nanomedicine strategies for livestock infections. Nanotechnology for Animal Health and Production. 61

Part III
Organ-Based Targeting

Chapter 4
Image-Guided Delivery of Therapeutics to the Brain

Lipa Shah, Arun K. Iyer, Meghna Talekar, and Mansoor M. Amiji

4.1 Challenges in CNS Delivery

Recent advances in CNS research have led to increased awareness and understanding of neurological disorders like pain, brain cancer, psychiatric disorders, and neurodegeneration. However, it still prevails to be one of the largest areas in pharmaceutical world, where there is an unmet medical need. Neurodegeneration, for example, comprises of many CNS diseases, such as Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's disease, Parkinson's disease, head trauma, epilepsy, and stroke. These disorders are devastating and expensive, with annual costs currently exceeding several hundred billion dollars in the USA alone, and current treatments are inadequate [1].

4.1.1 Therapeutics for CNS Diseases

CNS arena is quite challenging to treat and the current treatment options are not adequate, primarily due to the presence of physiological barriers, namely, the blood–brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier. Complex tight junctions (TJs) between the endothelial cells (ECs) of the brain, together with multiple transporters present on the luminal membrane of the EC, imparta

L. Shah • M. Talekar • M.M. Amiji (✉)
Department of Pharmaceutical Sciences, School of Pharmacy,
Northeastern University, Boston, MA, USA
e-mail: m.amiji@neu.edu

A.K. Iyer
Department of Pharmaceutical Sciences, Applebaum College of Pharmacy of Pharmacy,
Wayne State University, Detroit, MI 48201

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_4

Table 4.1 Bio-therapeutics for the treatment of CNS disorders

Bio-therapeutic	Disease	In vivo effect	Reference
Brain nanocapsules—epidermal growth factor receptor (BNCs—EGFR)	Brain tumor	Targeted delivery to brain tumor	[4]
Glycosylation of Tyr-d-Thr-Gly-Phe-Ser-NH_2	Pain	Improved bioavailability to the brain	[5]
Nerve growth factor (NGF)—gene therapy	Alzheimer's	NGF reduces cholinergic neuronal degeneration in patients with Alzheimer's disease	[6]
Brain-derived neurotrophic factor (BDNF)	Neuronal loss	Complete neuroprotection of hippocampal CA1 neurons in transient forebrain ischemia	[7]
Vasoactive intestinal peptide (VIP)		Increase in cerebral blood flow	[8]
Lenti-glial cell-derived growth factor (GDNF)	Parkinson's	Prevent nigrostriatal degeneration and induce regeneration in Parkinson's disease	[9]

formidable obstacle to drug entry. Alternate modes of drug delivery have gained quite some research interests among academia, government institutions and private industry. Potential to treat CNS diseases using bio-therapeutics, like the monoclonal antibodies, peptides, proteins, nucleic acids, etc. are being explored and Table 4.1 highlights few examples reported in the literature for several diseases of the CNS. These therapeutics have particular advantages of improved specificity, potency and reduced side-effects when compared to the classical small molecules [2] to treat CNS diseases of neurodegeneration, pain, psychiatric disorders, gliomas, etc. However, there aren't as many biologics that have been successfully developed in clinic for treating CNS disorders [3]. Lack of drug-like properties coupled with in vivo instability, poor penetration across the CNS, and cost of manufacture of biologics have limited their entry into market [2]. Passage of bio-therapeutics into the brain hence needs an effective delivery mechanism that can cross the BBB.

Current means to deliver therapeutics to the CNS can be broadly classified into invasive and noninvasive strategies; some of these are highlighted in Table 4.2 [1, 10]. Owing to the complexity of neurosurgical procedures like the intracerebroventricular and intrathecal injection or intracerebral implantation and the risks involved with invasive strategies; the noninvasive strategies are gaining more attention.

4.1.2 Major Issues in CNS Delivery

The unique physiology of brain presents multiple barriers to transport of many therapeutics, at the level of cerebral capillaries (i.e., the blood–brain barrier) and at the epithelia of the choroid plexus and circumventricular organs (i.e., the

Table 4.2 Invasive and noninvasive strategies for delivery to the CNS (reproduced with permission from ref. [10])

Administration strategies	Description and examples	References
Invasive strategies		
Intracerebroventricular injection	Intracerebral infusion or implant localized to a small area in the brain, e.g., tumor site, particular failing neuron. Clinical data reported with GDNF, CTNF neurotrophic factors	[11, 12]
Intra-arterial administration and transient disruption of the BBB	Intra-arterial administration increases drugs' systemic concentration by eliminating first-pass metabolism. Hyperosmolar sugar solutions (20 % mannitol) or immune adjuvants (Freund's) administration in carotid artery or application of ultrasound: shrinkage of endothelial cells, thereby causing transient opening of the tight junction (~20 min)	[13, 14]
Intraventricular/ intracerebral implants	Direct injection into the CSF; Subcutaneous implant in the scalp connected to the ventricles by a catheter (Convection enhanced drug delivery (CED). Intracerebral polymeric implants for local delivery	[15–17]
Focused ultrasound (FUS)	Utilizes microbubbles to create an acoustic cavity and a pressure shock wave that temporarily punctures the endothelial wall to cause a transient influx of otherwise impermeable drugs. Biotherapeutics like Rituximab, an anti-CD20 mAb, have been delivered across BBB using this methodology	[3, 18, 19]
Noninvasive strategies		
Altering the chemistry	Increasing the lipophilicity of molecules (e.g., prodrug approach), coupling nucleic acids to cell penetrating peptides, "molecular trojan-horse" approach are some of the techniques explored to improve BBB penetration	[20, 21]
Intranasal delivery	Likely that intranasally delivered therapeutics reach the CNS via the olfactory region or trigeminal pathway, bypassing the BBB, e.g., evidence of delivery of insulin or nerve growth factor (NGF) via intranasal drug delivery	[22, 23]
Colloidal (nanoparticle) drug carriers	Liposomes (e.g., NGF or immunoliposomes), emulsion (e.g., nanoemulsions), solid lipid nanoparticles (e.g., thiamine- coated nanoparticles) and polymeric nanoparticles (e.g., nerve growth factor using poly(butyl cyanoacrylate) nanoparticles) for delivery to the brain	[24]
Coadministration with inhibitors of efflux	Combination of anticancer drugs with specific P-gp inhibitors, like valspodar, zosuquidar, etc. lead to stronger P-gp inhibition and hence, better CNS penetration	[14]

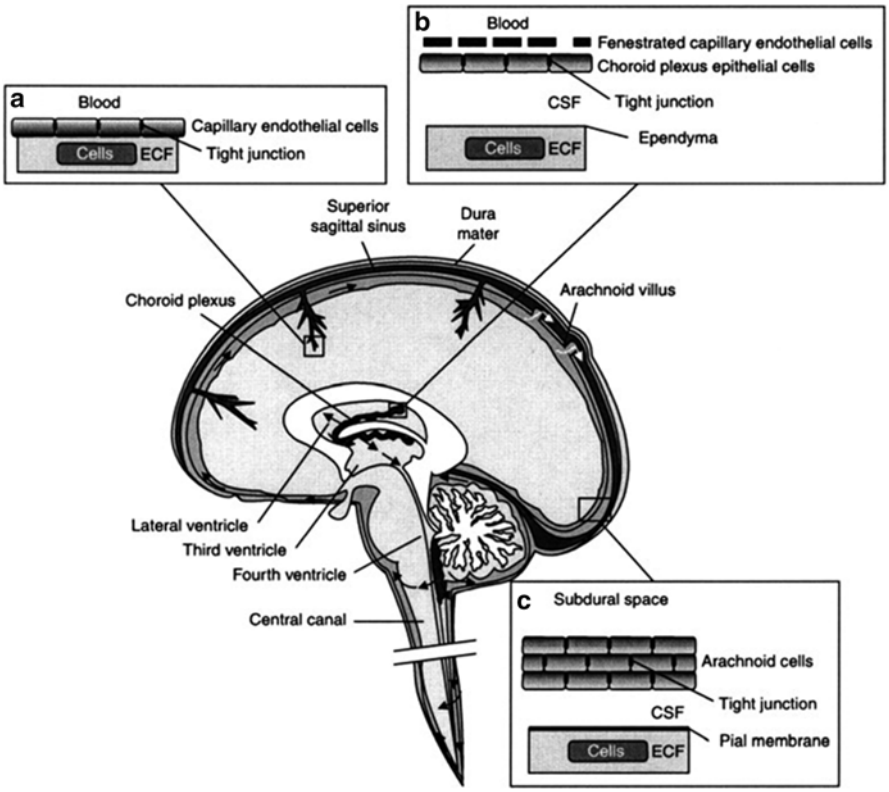

Fig. 4.1 Major physiological barriers to the CNS. (**a**) The blood–brain barrier (BBB) formed at the cerebral capillary endothelial cells by tight junctions. It encompasses the largest surface area, 12–18 m^2. Owing to a short distance of 25 μm between the brain cells and a capillary, the diffusion distances to neurons and glial cell bodies for solutes that cross the BBB are rather short, and hence, BBB is the favored route for targeted delivery of drugs to all brain cells. (**b**) The second prominent barrier, the blood–CSF barrier (BCSFB) lies at the choroid plexuses in the lateral, third, and fourth ventricles of the brain. Here, tight junctions are formed at the apical (CSF facing) surface of the endothelium. While some solutes enter the brain primarily via the choroid plexuses into CSF, some may enter via both the BBB and BCSFB. (**c**) The subdural space has the arachnoid barrier, which is formed by multilayered epithelium joined by tight junctions. The brain is enveloped by the arachnoid membrane, which is avascular and lies under the dura. A significant amount of CSF drains into the sinus, through the valve-like arachnoid villi which only allow a one-way CSF movement out of the brain to blood. Transport across the arachnoid membrane is not an important route for the entry of solutes into brain (Reproduced with permission from ref. [26])

blood–cerebrospinal fluid barrier) [25]. Figure 4.1 illustrates the common physiological barriers to CNS drug delivery [26].

The blood–brain barrier: BBB prevents 98 % of potential drugs from reaching their CNS targets. This has been a major obstacle to several therapies that are potentially useful for combating diseases that affect the CNS [27]. Macromolecules are unable to cross the capillary brain endothelial cells which form tight junctions at the

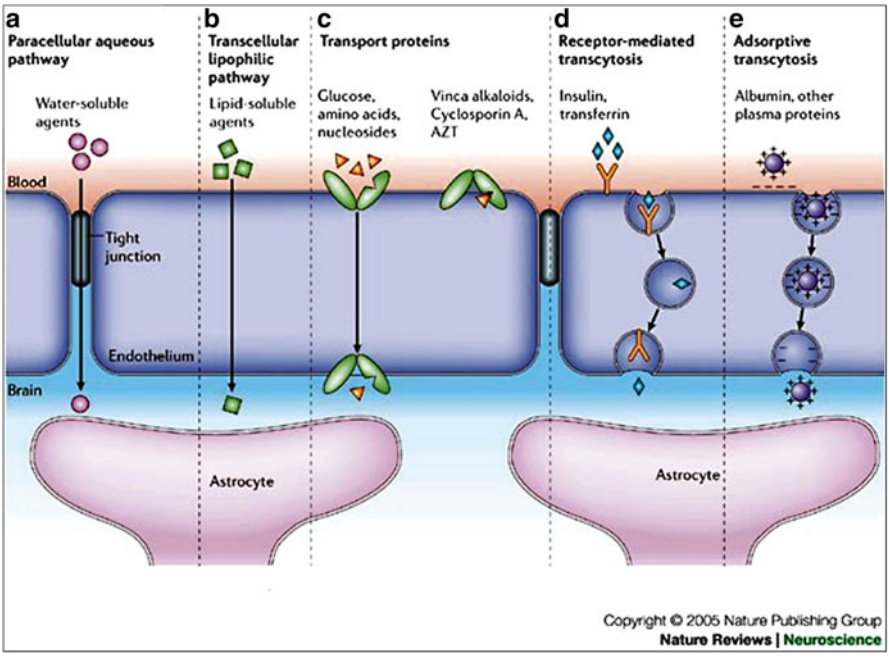

Fig. 4.2 A schematic representation of the blood–brain barrier (BBB) with endothelial cells in close proximity with the astrocytes, and known pathways for transport across the BBB. (**a**) Paracellular pathway: tight junctions restrict penetration of water-soluble agents. (**b**) Transcellular pathway: lipid bilayered endothelial membrane helps with diffusion of hydrophobic agents. (**c**) Transport proteins: The presence of transport carriers for glucose, amino acids, and other substances and, the energy-dependent efflux transporters at the endothelium. (**d**) Receptor-mediated: Specific receptor-mediated endocytosis and transcytosis result in transport of certain proteins, such as insulin and transferrin. (**e**) Adsorptive transcytosis: Uptake of native plasma proteins such as albumin. Pathways **b–e** are prominent in drug delivery across the BBB; most CNS drugs enter via route **b** (reproduced with permission from [29])

BBB. Figure 4.2 shows some of the pathways of drug and macromolecule transport across the BBB [28].

Due to presence of tight junctions at the cerebral microvasculature, predominant mechanisms for transport of therapeutics across the BBB are either by lipid-mediated free diffusion (which is restricted to lipophilic small molecules), or by use of endogenous uptake transporters present at the luminal side of the BBB [30]. Endogenous transport proteins mainly allow selective uptake of water soluble nutrients like glucose, amino acids or nucleosides; whereas, receptor-mediated transporters are involved in transport of large molecules like insulin, transferrin, etc. across the BBB [30]. Along with the influx transport proteins, there are also efflux transporters like the MDR1, BCRP, MRP1 present at the BBB which pump out the substrates from cells back into the bloodstream. A recent paper highlights the role of P-glycoprotein in predominantly causing efflux of antiepileptic drugs from the brain [31].

The blood–CSF barrier: This barrier exists between systemic circulation and the cerebrospinal fluid. It acts as a filter by allowing selective molecules through the cerebrospinal fluid. Unlike the BBB, blood–CSF barrier has a lot less surface area and hence, likely to pose less obstacle in drug delivery to CNS [1, 32]. However, it forms mechanical barrier between the epithelial cells of the choroid plexus, which prevents drugs from entering the CSF. There are fenestrae between the capillaries that supply this region, where the drugs and other molecules can freely diffuse, but they are prevented from entering the CSF due to the epithelial tight junctions.

Systemic distribution and clearance: Lipophilic compounds are often used for CNS therapy as they lead to increase in CNS permeability. These lipophilic compounds more readily penetrate into peripheral tissues in the body when administered systemically, and this causes significant dilution and metabolism of these drugs, hence requiring a larger dose to achieve the required therapeutic levels in the brain [7]. This leads to nonspecific systemic side effects. In addition, lipophilic compounds tend to have high plasma protein binding; which causes decrease in the free fraction of circulating drug available for therapeutic efficacy. This problem can be addressed by altering the release and distribution profile of therapeutics via their encapsulation in the novel drug delivery systems [1]. The discovery of antennapedia (Antp)-mediated transduction of heterologous proteins into cells, and of other "Trojan horse peptides," is an example for effective delivery of biological therapeutics across the BBB and cellular membranes [33].

4.2 Imaging Applications for CNS Diseases

Imaging plays a central role in the diagnosis of the disorders of central nervous system (CNS). Several different imaging modalities such as ultrasonography (US), optical imaging, X-ray computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI) have been employed for CNS imaging. Among them, MRI has by far been one the most widely used clinical imaging modality for the assessment of CNS diseases [2]. Computed tomography (CT) also continues to offer several advantages that are lacking in MR imaging. Similarly, ultrasound (US) imaging also compliments MRI and PET and achieves good spatial and temporal depth resolution that is lacking in other functional brain imaging modalities. Optical imaging is becoming increasingly popular for brain imaging due to exquisite sensitivity to functional changes through intrinsic contrast as well as ease of synthesis and availability of optical probes that can be used as exogenous contrast agents [3]. Most of the imaging techniques are capable of allowing for real time noninvasive imaging of the brain. More importantly the availability of advanced instrumentation and technological breakthroughs in multimodal imaging systems (such as PET-CT, MR-CT and PET-MR) provides great promise for the evaluation of several physiologic parameters [4–6]. For instance, regional cerebral perfusion can be evaluated by

intravenous infusion of contrast agents and performing perfusion-weighted MRI in conjunction with CT [34]. Similarly, in the case of patients with acute stroke disorders, MRI evaluation can provide very useful information such as very early changes in free water diffusion in the brain [35]. In addition, the ever-expanding field of functional MRI (fMRI) can be used to provide topographic plots of brain activity using blood flow and regional oxygen consumption in the brain [36]. Also, new techniques such as functional ultrasound (fUS), has the capability to image whole-brain microvasculature dynamics in response to brain activation with high spatiotemporal resolution [37]. Overall, it has become imperative to choose the imaging modality based on the indication of the disease, the strengths and weakness of all the available techniques, and the cost-to-benefit analysis for the patient. We discuss below some of the important modalities used for CNS imaging.

4.2.1 Magnetic Resonance Imaging (MRI)

MRI)is the one of the most preferred procedure for imaging CNS disorders [38]. In addition to offering a major advantage of imaging in any plane, MRI provides extremely good soft tissue contrast, which lacks in CT or ultrasound imaging. However, the most attractive feature of MR imaging is the lack of harmful ionizing radiation that is indispensable for nuclear modalities imaging such as CT, PET, or SPECT. Also, the artifacts seen in CT imaging such as beam hardening from the bone/base of the skull or dental implants are also absent in MR imaging [39, 40]. MRI is very useful to detect a variety of conditions of the brain such as development structural abnormalities [41], cysts [42], tumors [43], inflammation or bleeding due to injury to the brain [44]. In addition, it can also be used to detect infections, or problems with the blood vessels [36, 45]. MRI of the brain can be useful in evaluating disorders such as dizziness, persistent headaches, or seizures [46], and it can help to detect certain chronic diseases of the nervous system, such as multiple sclerosis [47, 48]. The disadvantage of MRI includes longer imaging times, increased artifacts due to patient motion, and increased cost of instrumentation. MRI may also be contraindicated in some cases because of the presence of certain implants or metallic foreign bodies [49].

4.2.2 X-Ray Computed Tomography

X-ray computed tomography (CT) has proved useful for evaluating CNS disorders, brain ischemia, and injury such as assessment of hemorrhage, edema, and fractures [50–52]. CT can provide several advantages over MRI which include short imaging times (about 1 s per slice), widespread availability, ease of access, optimal detection of calcification and hemorrhage (subarachnoid hemorrhage), and excellent resolution of bony detail [53]. Even today, CT remains a procedure of choice

in the evaluation of acute intra-cerebral hemorrhage because of its high sensitivity and easy access. CT is also valuable for patients who cannot be subjected to MRI because of implanted biomedical devices or ferromagnetic foreign material [49]. Disadvantages of CT include the use of ionizing radiation and frequent artifacts arising from dense bone/base of the skull [54]. Although CT may occasionally suggest the diagnosis of some CNS disease conditions, MR imaging demonstrates this abnormality to better advantage [55]. For instance hemorrhages of the corpus callosum and the midbrain are common, and MRI is more sensitive than CT in detecting the foci of edema and hemorrhage [56]. MRI can also can detect blood breakdown products (hemosiderin) and thus is helpful in evaluating remote traumatic brain injury [57]. Thus in several cases, multimodal anatomic (such as CT) and functional imaging (such as MRI) can provide better information for evaluating CNS disorders.

4.2.3 Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) Imaging

Among the nuclear modality imaging, PET having high detection sensitivity has been one of the most sought instrumental techniques. Indeed, PET can be considered as one of the most powerful molecular imaging technique currently available for general clinical management of several disease conditions [58]. PET has been widely used in the study of several CNS disorders [59]. A number of different molecular markers labeled with positron-emitting isotopes, such as fluorine-18 ([^{18}F], carbon-11 ([^{11}C]), oxygen-15 ([^{15}O]), and nitrogen-13 ([^{13}N]), have been developed for measuring cerebral blood flow (CBF), neurotransmitter system and cerebral metabolism [1, 2]. Almost every aspect of brain physiology can in principle be evaluated by using PET radiotracers. In this regard, one of the most well-known and widely used PET radiopharmaceutical for both research and clinical application is fluorine-18 labeled fluorodeoxyglucose (^{18}F-FDG) (with a half-life of ~110 min and high-energy positron emission (0.6335 MeV) [60]. FDG-PEt allows for the evaluation of glucose metabolism and has physical characteristics that make it relatively easy to produce and use. FDG-PET has been frequently used for staging of several solid tumors [61, 62]. In addition, FDG-PET provides high-resolution images of cerebral metabolism [63]. PET, in conjunction with PET probes have been utilized to study and mange many diseased states in the brain. In fact, PET was almost exclusively utilized for brain imaging in early clinical research, which provided huge amounts of data that have advanced the understanding of brain function. The most specific CNS disorders in which PET studies may influence the management of patients include brain tumors, seizures and movement disorders, dementia, and head trauma [58, 63–70]. There are some disadvantages of PET probes. The production of positron emitting PET nuclides generally requires a dedicated and costly cyclotron in close proximity to the imaging facility due to the short half-lives of radionuclides. It is for this reason that radiolabeling drugs/molecular markers with

PET-radiotracers are at times difficult to produce and image within the time frame of their decay [71]. In this regard, ^{68}Ga can be used valuable alternative to ^{18}F for PET imaging because it does not need an on-site cyclotron and also because of its high positron emission of 1.899 MeV [71, 72].

Another alternative to PET is the use of SPECT imaging. Although SPECT generally has lower radionuclide detection efficiency over PET, recent advancements in multi-aperture pinhole SPECT technology significantly reduces the difference in detection efficiencies between both modalities [73]. ^{99m}Technetium (^{99m}Tc) is a versatile radioisotope for SPECT imaging that emits readily detectable 140 KeV γ-rays with a half-life of ~6 h [74]. The time frame of its decay is ideally suited for labeling for in vivo setting because it is long enough for scanning with SPECT instrument but at the same time keeps the radiation exposure low and helps reduce radiation burden to patients [74]. ^{99m}Tc is thus currently the most commonly used isotope for several disease diagnosis including cancers [75]. In one recent study ^{99m}Tc was conjugated to human serum albumin ([^{99m}Tc]-HSA) for performing SPECT/MRI to study the role of inflammation in the cavernous sinus of cluster headache patients [76]. In another study SPECT using ^{67}Ga radiotracer was used successfully in patients with suspected CNS lymphoma [77]. Kungh et al. used iodine-125 labeled idobenzamide ([^{125}I]-IBZM) for the in vivo SPECT imaging of CNS D2 receptors in the brain. The imaging study revealed that ([^{125}I]-IBZM showed specific localization in the basal ganglia of the brain [78]. In yet another recent study, SPECT scans were used as an objective measure of abnormalities present in the patients brain in chronic Lyme disease, that frequently involves the central nervous system [79].

In the last few years, along with the increased interest in novel radiopharmaceutical (probe) development for CNS diseases, there has been a burgeoning growth in the development of cross-functional imaging technologies that are revolutionizing the field of biomedical imaging for disease management [80]. For instance, CT or MRI that can provide structural information is being combined with radionuclide imaging techniques such as PET/SPECT or optical imaging that lacks these features [81]. Some such examples include the clinical hybrid/fused PET-CT and SPECT-CT [82] and preclinical small animal micro-PET/SPECT-CT imaging systems, which has the ability to co-register both functional and anatomical features previously lacking in SPECT or PEt alone [83]. Other combinations of functional and structural imaging modalities such as PET/MRI and SPECT/MRI are also used both for preclinical and clinical imaging [76, 84–86].

4.2.4 Noninvasive Optical Imaging

Noninvasive optical imaging of the brain was demonstrated more than three decades ago, in which near-infrared (NIR) light was used for measurement of changes in blood flow and tissue oxygenation in the brain [87]. Since then, there has been tremendous growth in the area of optical imaging for preclinical and clinical

imaging of the brain [88–93]. There are distinct advantages of using light to image tissues. Apart from the instrumental advantage (such as relatively ease in access and cost of instrumentation) and the nonionizing nature of light used, the most attractive feature of optical imaging lies in its ability to provide excellent sensitivity to functional changes that occur due to intrinsic changes in absorption, fluorescence, or scattering of the (intrinsic) chromophores. The most commonly used intrinsic chromophores are oxyhemoglobin and deoxyhemoglobin (HbO_2 and HbR). However, there has also been an increased interest in extrinsic NIR contrast agents for preclinical and clinical imaging [94, 95]. The most challenging aspect of optical imaging is to overcome the effects of scattering of light, which generally limits the penetration depth and achievable imaging resolution. Although light penetration and scattering pose as obstacles for performing optical imaging in adult humans, it has been easier to achieve in children and babies. Optical imaging provides a unique opportunity because other techniques, such as function MRI (fMRI) and PET, are difficult to perform on infants and babies. Moreover, the studies in babies are motivated due injuries to the brain caused by premature birth, birth asphyxia, and congenital conditions [96]. It also allows for assessing infant brain development that has been previously inaccessible by other imaging modalities [97]. However, in the case of adults, optical imaging can complement other imaging modalities such as fMRI by providing additional functional information [98]. Preclinical noninvasive optical brain imaging has been performed on animals in general as a precursor to clinical imaging in humans or for longitudinal developmental studies [93, 99, 100].

4.2.5 Illustrative Examples of CNS Imaging

Although MRI, CT, PET, SPECT, and optical imaging have been used individually for imaging a number of CNS conditions recent focus has been on the development of systems, which enable multimodal imaging. Kircher et al. recently reported the development of a triple-modality magnetic resonance imaging, photoacoustic imaging, and Raman imaging (MPR) using nanoparticle system which provided enhanced brain imaging allowing accurate demarcation of tumor and healthy tissue margins [101]. MPR enhanced the MRI contrast-to-noise ratio from 2.2 ± 0.3 to 14.0 ± 1.9, increased the photoacoustic signal by 75 % and also provided Raman signal post-injection of the MPR nanoparticle (Fig. 4.3). The triple modality system also enabled accurate tumor imaging and surgical resection thus indicating the benefit of combining multiple imaging modalities [101].

The concept of multimodality imaging was also explored by Fan et al., who investigated development of multifunctional microbubble (MBs) system loaded with doxorubicin and conjugated with superparamagnetic iron oxide nanoparticles (SPION) [102]. MBs are gas bubbles (air or perfluorocarbon) encapsulated in a stabilizing shell (albumin or lipid) and are used as contrast agents in ultrasound based imaging. When these MBs are administered in vivo and exposed to ultrasound they

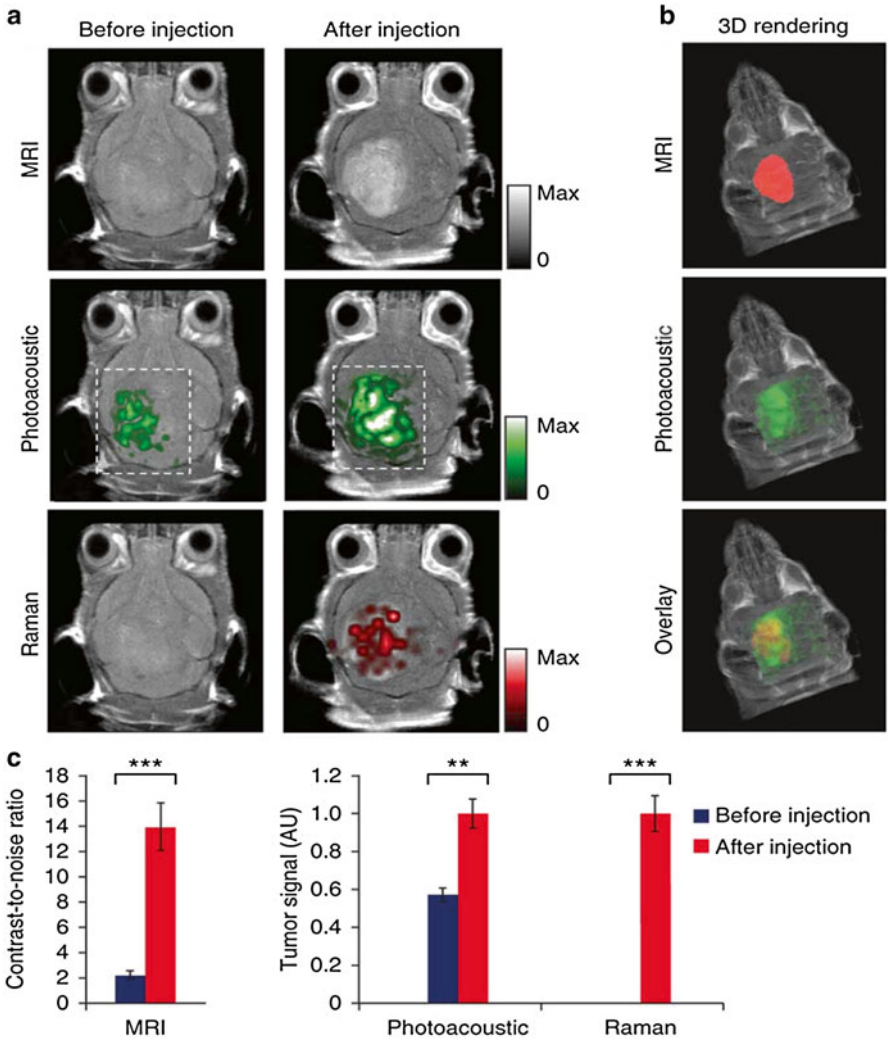

Fig. 4.3 Multi-modality imaging of brain tumors in living mice using MPR nanoparticles. (**a**) Two-dimensional (2D) axial MRI, photoacoustic and Raman images. The post-injection images of all modalities showed clear tumor visualization (*dashed boxes* outline the imaged area). (**b**) A three-dimensional (3D) rendering of MRI with the tumor segmented (*red*; *top*), an overlay of the 3D photoacoustic images (*green*) over the MRI (*middle*) and an overlay of MRI, the segmented tumor and the photoacoustic images (*bottom*) showing good co-localization of the photoacoustic signal with the tumor. (**c**) Quantification of the signals in the tumor showing a significant increase in the MRI, photoacoustic, and Raman signals after as compared to before the injection. $n = 4$ mice. Data represent mean ± s.e.m. $^{***}P < 0.001$, $^{**}P < 0.01$ (one-sided Student's t test.) AU, arbitrary units (reproduced with permission from ref. [101])

expand and contract at the frequency of the propagating acoustic wave. Once these oscillations are above a certain threshold these microbubbles collapse, inducing high temperature and pressure, which results in a shock wave that can puncture vessel walls including the blood–brain barrier (BBB). Following disruption by these MBs, the BBB can remain permeable for hours, allowing accumulation of therapeutic agents in the brain. SPIONs (size 50–180 nm) on the other hand are MRI contrast agents often used to evaluate BBB dysfunction, cerebrovasculature perfusion, and in vivo cellular tracking. The SPION based multimodal system designed by Fan et al. not only delivered doxorubicin to the desired site but also provided image guided delivery of the cytotoxic agent to the tumor tissue.

Like the SPION, ultrasmall paramagnetic iron oxide nanoparticles (USPION) (size 10–50 nm) have been developed for the purpose of MRI imaging. Wadghiri et al. recently reported the development of USPIO-poly (ethylene) glycol (PEG)-Ab 1-42 nanoparticles to detect amyloid β plaques in Alzheimer's disease [103]. Traditionally in Alzheimer's disease PET imaging is used to visualize amyloid plaques in early stages of the condition when effective therapy can be initiated in patients. However, due to low spatial resolution of PET imaging many plaques cannot be imaged effectively. In comparison to PET and optical microscopy, MRI would provide effective amyloid plaque detection especially in the early stages due to MRI's high spatial resolution. Chen et al. had previously reported the binding of Ab 1-40 peptide with gadolinium diethylenetriaminepentaacetic acid (Gd-DPTA) or monocrystalline iron oxide nanoparticles (MION) for MRI imaging. The system was delivered via the intra-carotid artery along with mannitol to enhance BBB permeability. However, despite being able to use MR for imaging only mature plaques could be visualized. Following subsequent studies the USPIO-PEG-Ab 1-42 nanoparticles were developed which contained USPIO to enable intravenous injection, PEG to enhance BBB permeability and Ab 1-42 to target the nanoparticles to amyloid β plaques. Based on their work it was observed that this multimodal system enabled effective BBB penetration, targeting of the nanoparticle to the amyloid β deposits and MRI imaging could be used to detect these amyloid β plaques in Alzheimer's disease transgenic mice. Thus the finding of this work provides an opportunity of developing and evaluating novel therapeutic approaches to reduce the amyloid deposits in Alzheimer's disease.

Many of the CNS imaging techniques are often focused on the imaging of diseased tissue such as dopaminergic imaging in Parkinson's disease [104], molecular imaging of microglia and macrophages in the brain to understand neuroinflammation [105], and diagnosis of CNS viral infections such as encephalitis, meningitis, encephalomyelitis, and encephalomyeloradiculitis [106]. Likewise these conventional imaging techniques have also been used for cell tracking especially for tracking of stem cells transplanted for the treatment of cerebral conditions such as stroke, spinal cord injuries and central nervous system degenerative conditions such as Alzheimer's and Parkinson's disease to identify and monitor fate of particular groups of cells. Successful cell tracking has been carried out by combining MRI with SPION's [107–109], optical imaging with quantum dots [110, 111], or PET with unstable β+nuclides [112]. MRI is thought to be an excellent tool for in vivo CNS imaging; however, for effective MRI the cells are required to be labeled with a contrast agent, which would enable distinction between stem cells and in vivo

background. Several commercially available contrast agents have been investigated for imaging stem cells; however, due to their low internalization efficiency, dilution post administration in vivo, and rapid degradation, long term stem cell tracking has been stalled [113]. In order to overcome this issue and to provide a biological safe system, Wang et al. recently reported the development of SPIO@Au nanoparticles which were labeled to stem cells and post injection in mice could be traced for a period of 1 month [114]. Yi et al. reported the development of Fe_3O_4@SiO_2 core shell nanoparticles which were used to label human mesenchymal stem cells (hMSC) and were imaged using MR imaging [115]. The authors observed that in vivo imaging of the Fe_3O_4@SiO_2 labeled hMSC provided enhanced MRI imaging compared to unlabeled hMSC. This idea of imaging mesenchymal stem cells was extended further by Huang et al., who prepared mesoporous silica nanoparticles with FTIC, NIR dye ZW800, Gd^{3+}, and ^{64}Cu which were loaded into mesenchymal stem cells, enabling multimodal imaging using optical imaging, MRI, and PET [116]. Optical microscopy enabled visual monitoring of interaction between mesoporous silica nanoparticles and mesenchymal stem cells. MRI provided information about the distribution of the mesenchymal stem cells in the tumor region while PET helped to understand the dynamics between the system and in vivo condition and also provided a mechanism to quantify targeted delivery of the nanoparticles (Fig. 4.4). Further to this it was also observed that the multifunctional nanoparticle system could be integrated into the mesenchymal stem cells without inducing toxicity or affecting intrinsic cellular functions.

4.3 Image-Guided Therapeutic Delivery

The advances in imaging technologies in recent years have opened up an arena of image-guided delivery of therapeutics. While number of compounds emerging from drug discovery for the treatment of CNS disorders is expanding, a common challenge that is being faced is their inadequate delivery to the CNS. The most commonly practiced routes of delivery, namely the systemic and intrathecal administration have certain drawbacks [117]. Systemic delivery is often faced with systemic toxicity, non-targeted drug disposition and is impacted due to the inability of compounds to cross the blood–brain barrier [118, 119]. Intrathecal or intraventricular delivery is based on diffusion and these results in non-targeted heterogeneous dispersion of the drug in the CNS [117]. Hence, alternate forms of drug delivery, especially those that are guided to the site of interest via imaging are attractive.

4.3.1 Convection-Enhanced Drug Delivery

Convection-enhanced drug delivery (CED) has been explored to deliver small and large molecule therapeutics to the CNS. This technique involves invasive delivery of molecules, via a cannula into the interstitial spaces of the CNS tissues and hence

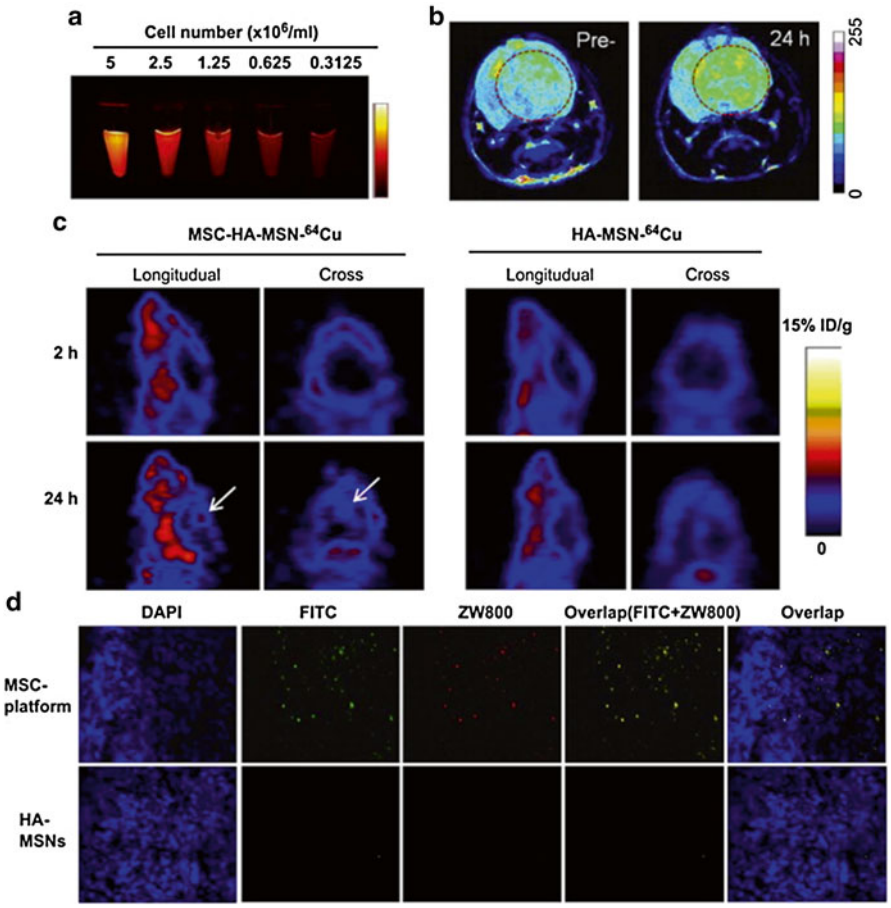

Fig. 4.4 In vivo multimodal imaging of the tumor targeting of the MSC-nanoparticles to orthotopic U87MG glioblastoma. (**a**) The near-infrared fluorescence signal of MSCs labeled with MSN and HA-MSN particles varied with an increase in cell concentration. (**b**) MR imaging demonstrated the increased signal at the tumor sites after MSC-nanoparticle administration for 24 h compared with pre-injection. (**c**) PET imaging of the tumor targeting of the MSC-nanoparticle and HA-MSN-^{64}Cu at the indicated time points. (**d**) Frozen tissue slices confirmed that the tumor uptake of the MSC-nanoparticle was significantly higher than free HA-MSNs, implying targeting of the MSC-nanoparticle to tumors (reproduced with permission from ref. [116])

bypassing the blood–brain barrier. Factors influencing tissue distribution of the drug by CED include drug half-life and tissue binding properties, drug flow-rate and volume; as well as the size, shape, and placement of the cannula [120, 121]. The work done by Lonser and colleagues for the treatment of neuronopathic Gaucher disease highlights a very promising application of combining CED, for delivering glucocerebrosidase coupled with, real-time monitoring of enzyme distribution using MRI tracer (gadolinium diethylenetriaminepenta-acetic acid [Gd-DTPA]) [117]. While CED allows stereotactic placement of drug into the brain to allow penetration

through a larger volume of the tissue, when compared to diffusion- based delivery, it does not alter the drug release from the vehicle delivered [122]. Hence, numerous research publications are combining nanoparticulates as drug delivery to the brain using CED as the mode of delivery.

While CED based drug delivery to the brain shows promise in delivering therapeutics to the brain, it suffers from a major setback or requiring surgical procedures and potential to cause high intracranial pressure [123].

4.3.2 Focused Ultrasound for BBB Disruption

Intra-arterial injection of certain hyperosmotic solutions such as mannitol, leads to transient opening of the tight junctions of endothelial cells of the BBB within the entire tissue volume supplied by the injected artery branch. Therefore, this phenomenon lacks the ability to selectively target a particular region in the brain that may be affected [124]. Patrick et al. [125] studied disruption of the blood–brain barrier using high intensity focused ultrasound. However, such technique is often associated with tissue damage, commonly the thermal induced lesions [126]. Hynynen and colleagues demonstrated for the first time [127] that, a low-power focused ultrasound applied after intravenous administration of a contrast agent and monitored via magnetic resonance imaging (MRI), induced a reproducible, focal blood–brain barrier opening by using intravascular gas bubbles as cavitation sites. The induction of these bubbles allowed reduction in the temporal average spatial peak powers by about two orders of magnitude and hence, no obvious permanent damage to the brain tissue was seen [127]. Also, following this technique of MRI-targeted focused ultrasound induced disruption of BBB, an intravenously administered dopamine receptor-targeting antibody crossed the BBB and recognized its antigens [19]. Figure 4.5 shows the T1 and T2 MRI of mouse brain after subjecting the hippocampus to ultrasound at 2.7 MPa and followed by an injection of contrast enhancer, gadolinium [128].

While both of these techniques have demonstrated improved delivery of therapeutic to the brain guided by imaging, they are invasive and hence, alternate formulation strategies based on colloidal nanoparticulates have been studied.

4.3.3 Nanoparticulate Drug Delivery

Transcellular uptake, receptor-mediated endocytosis and active targeting are the major pathways for nanotechnologies to show improved drug delivery to the CNS. Various other strategies, like improving paracellular permeability across the tight junctions of the BBB by use of vasoactive agents, which momentarily permeabilize the blood vessel, have also been reported for drug delivery to the CNS. However, these are associated with significant side effects and dose limitation [129]. Use of hypertonic solution of mannitol, which transiently opens the tight junctions

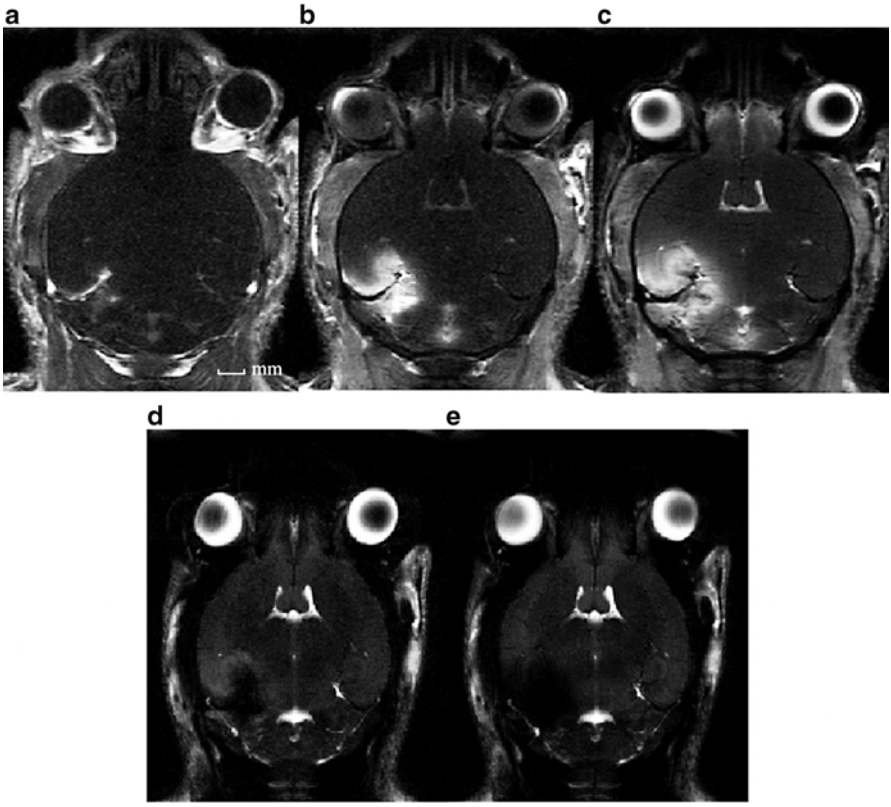

Fig. 4.5 T1and T2 magnetic resonance imaging (MRI) scans of a single mouse brain (approximately 3 mm beneath the top of the mouse skull). Ultrasonication at a pressure amplitude of 2.7 Mpa was applied and images taken. The ultrasound beam was focused onto the left hippocampus, while the right hippocampus was not targeted and hence served as a control. (**a**) 10 min after gadolinium (Gd) injection: Gd began to leak through the posterior cerebral artery near the hippocampus. (**b**) 35 min after Gd injection: Permeation of Gd through the hippocampus region showed an increase. (**c**) 95 min after Gd injection: Gd showed complete permeation throughout the hippocampus. (**d**) and (**e**): T2 MRI scans were obtained 20 min (**d**) and 50 min (**e**) after Gd injection, shows a suppressed signal where gadolinium is released. This corresponds to the regions of contrast enhancement seen on the T1 MRI scans in (**b**) and (**c**) (reproduced with permission from ref. [128])

due to shrinkage of endothelial cells, thereby improving the penetration of therapeutics administered by intra-arterial infusion [130]. Another approach that is being explored is intranasal delivery of nanocarriers. This route allows to bypass BBB by crossing the olfactory epithelium to achieve direct nose-to-brain delivery via the olfactory or trigeminal nerve system [131]. Table 4.3 shows examples of several clinical trials of polymeric nanocarriers, implants and microchips [132].

Following intravenous administration, the colloidal systems, like liposomes, solid lipid nanoparticles, nanoemulsion, albumin nanoparticles, and polymeric nanoparticles can extravasate into compromised BBB, e.g., the brain tumor, which leads to a more selective drug delivery into brain tumors [133, 134].

Table 4.3 Clinical trials of polymeric implants, microchips, and nanocarriers (adapted with permission from ref. [132])

Description of technology	Disease conditions	Phase/status	Trial identifier
O6-Benzylguanine and Carmustine implants in treating patients with recurrent malignant glioma	Brain and central nervous system tumors	Completed	NCT00004892
Phase IIa safety and light dose-escalation study in patients with primary or recurrent/high-grade glioma using the LitxTM system to confirm the zone of tumor destruction during the intraoperative treatment of glioma	Glioma, glioblastoma multiforme, anaplastic astrocytoma	Completed	NCT00409214
Examination of changes on Magnetic Resonance Imaging (MRI) in patients who receive Gliadel wafers during initial surgery for glioblastoma multiforme. Response or failure to Gliadel wafers for subjects with glioblastoma multiforme	Glioblastoma multiforme, anaplastic astrocytoma, anaplastic oligodendroglioma	Terminated	NCT00645385
Gliadel wafer and fluorescence-guided surgery with aminolevulinic acid followed by radiation therapy and Temozolomide in treating patients with primary glioblastoma	Brain and central nervous system tumors	Recruiting	NCT01310868
A study of intraventricular liposomal encapsulated Ara-C (DepoCyt) in patients with recurrent glioblastoma	Glioblastoma multiforme, glioma, astrocytoma, brain tumor	Recruiting	NCT01044966

An interesting application of combining CED with nanoparticle drug delivery for GBM therapy was via coupling of GBM-specific antibody (EGFRvIIIab) to the MRI-contrast enhancing magnetic iron oxide nanoparticles (IONPs) [135, 136] as illustrated in Fig. 4.6. Magnetic nanoparticles have a potential multifunctional advantage of providing cancer cell detection by MRI (contrast enhancement) as well as targeted cancer cell therapy [135]. These IONPs were administered using CED for targeted MRI imaging. The coupled antibody selectively bound to the epidermal growth factor receptor deletion mutant on human GBM and lead to significant improvement in median survival (19 days) in highly tumorigenic mice when compared to untreated group (11 days survival) [135, 136].

4.3.4 Illustrative Examples of Image-Guided Therapeutic Delivery

Targeted therapeutic delivery to the brain for treating several disorders is well explored to overcome the disadvantage of systemic delivery of such therapeutics, which often suffers from lack of passage through the BBB. A paradigm in CNS

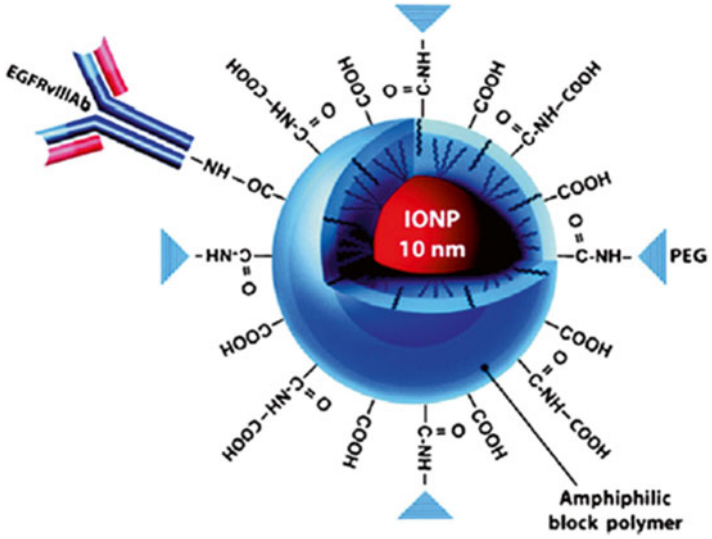

Fig. 4.6 Representation of an amphiphilic block polymer-coated magnetic iron oxide nanoparticle (IONP). The core is IONP (*red color*, size 10 nm) coated with a biocompatible amphiphilic copolymer bioconjugated to the GBM-specific antibody (EGFRvIIIab). Polyethylene glycols (PEG) are present on the surface of the polymer for stabilization of these IONP. Bioconjugation of the EGFRvIIIab is performed to the carboxylic (–COOH) group of the polymer coating of the IONP (reproduced with permission from ref. [136])

therapy would be to combine therapeutic delivery with imaging modality. An example of utilizing such an approach for controlling distribution of glucocerebrosidase enzyme in the region of brain and brainstem, in patient with neuronopathic Gaucher disease, has been translated in clinic [117]. Perfusion of glucocerebrosidase to clinically affected anatomic sites (right facial (VIII) and abducens (VI) nerves within the brainstem nuclei), coupled with real-time MRI monitoring revealed progressive and complete filling of the targeted region with enzyme and gadolinium diethylenetriamine penta-acetic acid (Gd-DTPA) infusate [117] (Fig. 4.7). Thus, convection-enhanced delivery can be used to safely perfuse large regions of the brain and brainstem with therapeutic levels of glucocerebrosidase. The authors extend the application of this technology towards treatment of other CNS disorders like, metabolic storage diseases, tumors and degenerative diseases [137].

Fig. 4.7 (continued) (pre-operative), targeted for perfusion (*green-shaded area*). Co-infusion of a surrogate MRI tracer with glucocerebrosidase enzyme, ensured its accurate and adequate distribution to the targeted region. Corresponding intraoperative, T1-weighted MRI in the sagittal (**e**) and coronal (**f**) planes after delivery of 1.8 mL of gadolinium diethylenetriamine penta-acetic acid (Gd-DTPA) and glucocerebrosidase into the targeted region in the pons (distribution of Gd-DTPA is the *white area*) (reproduced with permission from ref. [117])

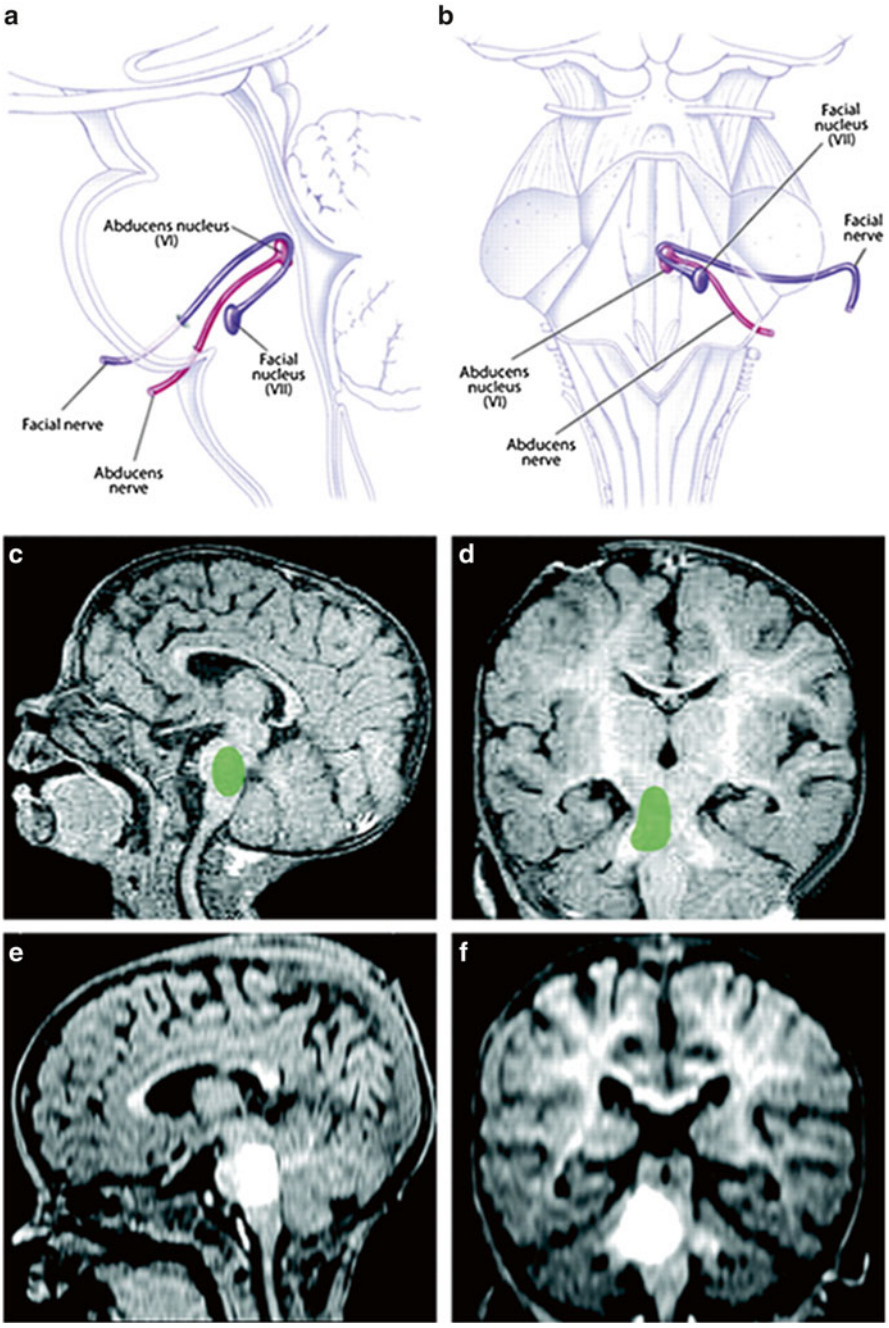

Fig. 4.7 Schematic views of the brainstem, illustrating the anatomic location of affected right VII and VI cranial nerves in patient with Gaucher disease, in sagittal (**a**) and coronal (**b**) planes. Also, T1-weighted MRI corresponding region in the sagittal (**c**) and coronal (**d**) planes of brainstem

Sawyer et al. were the first to demonstrate a combination-based therapy of intracranial tumors utilizing polymer based camptothecin (CPT) nanoparticles with CED to allow delivery to a stereotactically defined location in the brain for long period of time [122]. While, all of the control rats (untreated) died from progressive tumors by 16 days after implantation, drug treated rats showed improved survival, and group with CED of nanoparticles had significantly more long-term survivors (30 %) and improved median survival (22 days) when compared to blank nanoparticles [122]. Figure 4.8 illustrates the histopathology and fluorescent images of the intracranial CED- based administration of either drug-loaded nanoparticles or blank nanoparticles, or unencapsulated CPT nanoparticles [122]. One of the reasons for improved survival of rats and disappearance of gliomas in this study is likely due to increased volume coverage of drug owing to CED- based administration. This technology has the potential to apply to clinic for wide range of insoluble cancer therapeutics, to improve the delivery and reduce the dose thereby, reducing the toxicity of these drugs.

Thus, image-guided targeted delivery of therapeutics to the brain using invasive techniques like focused ultrasound or convection-enhanced drug delivery has shown tremendous benefit and improvement in treating CNS disorders. The concept has proven application in a clinical setting. Combination of nanoparticulate drug delivery with an imaging component is a promise to improve the targeting of therapeutics to the CNS.

4.4 Conclusions

The delivery of therapeutics to the brain requires traversing the highly impermeable BBB which has remained one of the greatest challenges for the successful treatment of disorders of the CNS. Although current regimens using naked antibodies, peptides and drugs have shown progress in treating such conditions, their complete utility remains unexplored in the absence of appropriate delivery systems. In recent times, there has been a burgeoning interest among researchers and clinicians to explore novel invasive and noninvasive delivery technologies due to the tremendous potentials offered by such interventions in overcoming the delivery barriers to the brain, and in exploring new paradigms to effectively resolve disorders of the CNS. In this regard, there is also growing interest in utilizing imaging technologies in conjunction with therapeutic interventions that can not only aid in diagnosis of disorders but also assist in monitoring the progression of disease, as well as for measuring treatment response. Among the imaging modalities, techniques such as MRI and X-ray CT have been employed in the clinics at large. In recent times, radionuclide imaging using SPECT and PET and optical imaging using fluorescent probes have also shown tremendous utility in preclinical and clinical brain mapping. The combinations of cross-functional imaging systems such as PET/MRI, PET/CT and SPECT/CT are becoming increasingly popular due to their ability to noninvasively image

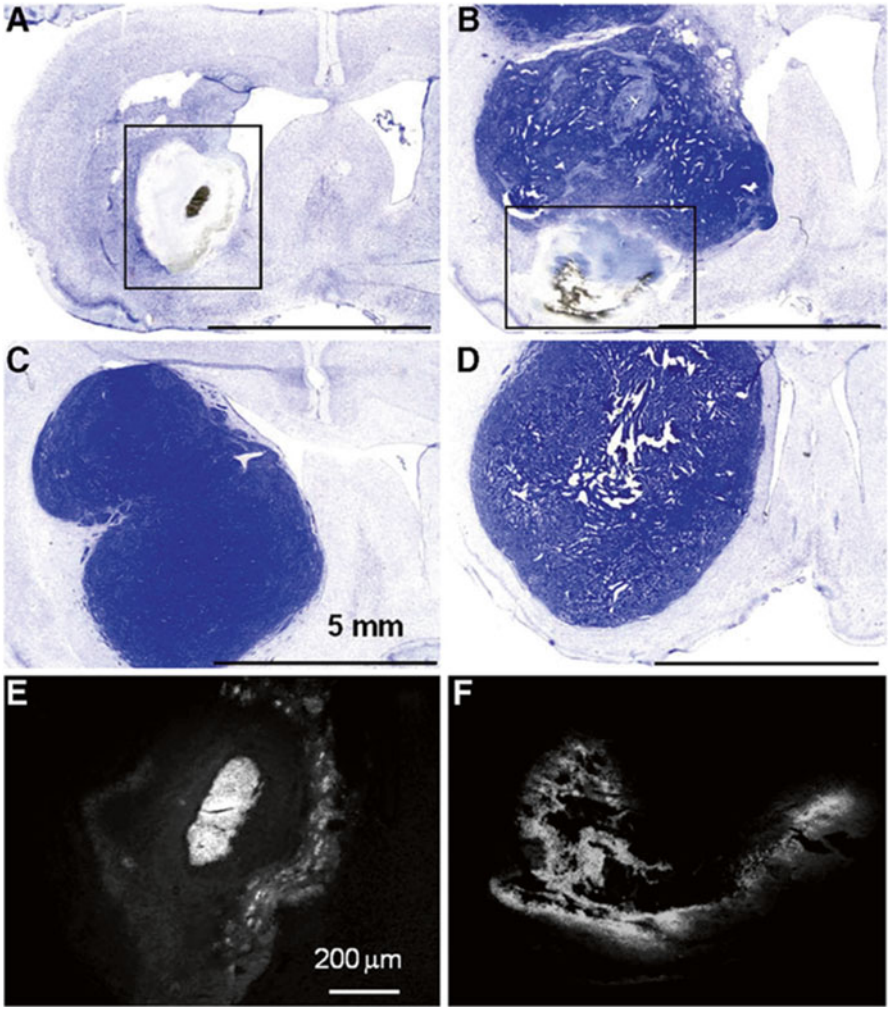

Fig. 4.8 Histopathology of brains isolated from an orthotropic glioma model in rat, following convection-enhanced delivery (CED) based administration of camptothecin (CPT) nanoparticles (**a**, **b**) or free drug (**c**) or blank nanoparticles (**d**): After tissue harvesting, cresyl violet was used to visualize the remaining tumor. **a**: Rats that survived for 60 days, i.e., until the end of the study, showed no remaining tumor. A cavity was formed at the site of the tumor location. **b**: Some rats that received a full dose of nanoparticles died prior to the end of the study (at day 20). In both sets of rats (surviving and dead), residual nanoparticles delivered by CED were often visible (see *boxes* in **a** and **b**). **c**: Rats given CED of free drug or d: rats given CED of blank particles had large tumors with evidence of a midline shift and altered ventricles due to the presence of the tumor. These animals died at day 17 (**c**) and day 15 (**d**). **e** and **f**: Fluorescent images of sections shown in panels **a** and **b** reveal CPT (inherently fluorescent molecule) due to persistence of encapsulated in the tissue (reproduced with permission from ref. [122])

both molecular signatures and structural details of the brain. Thus, the concept of combining imaging and therapeutic interventions using a single nano delivery system or the so-called "theranostic" nanosystems is currently evolving as one of the most effective ways to compliment the diagnostic regimen with a therapeutic end goal, with the added advantage of progressive noninvasive evaluation of treatment response. Engineering of such multifunctional nanoparticles that can simultaneously perform multiple roles such as targeted payload delivery and multimodal imaging functions have shown great potentials in brain delivery/imaging, unraveling promising avenues for improving the outcome of treating CNS disorders in the clinic.

References

1. Barchet TM, Amiji MM (2009) Challenges and opportunities in CNS delivery of therapeutics for neurodegenerative diseases. Expert Opin Drug Deliv 6(3):211–225
2. McGonigle P (2012) Peptide therapeutics for CNS indications. Biochem Pharmacol 83(5):559–566
3. Rajadhyaksha M et al (2011) Current advances in delivery of biotherapeutics across the blood–brain barrier. Curr Drug Discov Technol 8(2):87–101
4. Tsutsui Y et al (2007) Development of bionanocapsules targeting brain tumors. J Control Release 122(2):159–164
5. Egleton RD et al (2001) Improved blood–brain barrier penetration and enhanced analgesia of an opioid peptide by glycosylation. J Pharmacol Exp Ther 299(3):967–972
6. Tuszynski MH (2002) Growth-factor gene therapy for neurodegenerative disorders. Lancet Neurol 1(1):51–57
7. Pardridge WM (1999) Vector-mediated drug delivery to the brain. Adv Drug Deliv Rev 36(2–3):299–321
8. Wu D, Pardridge WM (1996) Central nervous system pharmacologic effect in conscious rats after intravenous injection of a biotinylated vasoactive intestinal peptide analog coupled to a blood–brain barrier drug delivery system. J Pharmacol Exp Ther 279(1):77–83
9. Kordower JH et al (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290(5492):767–773
10. Shah L, Yadav S, Amiji M (2013) Nanotechnology for CNS delivery of bio-therapeutic agents. Drug Deliv Transl Res 3:336–351
11. Strasser JF et al (1995) Distribution of 1,3-bis(2-chloroethyl)-1-nitrosourea and tracers in the rabbit brain after interstitial delivery by biodegradable polymer implants. J Pharmacol Exp Ther 275(3):1647–1655
12. Bloch J et al (2004) Neuroprotective gene therapy for Huntington's disease, using polymer-encapsulated cells engineered to secrete human ciliary neurotrophic factor: results of a phase I study. Hum Gene Ther 15(10):968–975
13. Schenk D et al (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400(6740):173–177
14. Laquintana V et al (2009) New strategies to deliver anticancer drugs to brain tumors. Expert Opin Drug Deliv 6(10):1017–1032
15. Dahm P et al (1998) Efficacy and technical complications of long-term continuous intraspinal infusions of opioid and/or bupivacaine in refractory nonmalignant pain: a comparison between the epidural and the intrathecal approach with externalized or implanted catheters and infusion pumps. Clin J Pain 14(1):4–16
16. Krewson CE, Klarman ML, Saltzman WM (1995) Distribution of nerve growth factor following direct delivery to brain interstitium. Brain Res 680(1–2):196–206

17. Ilias W, Todoroff B (2008) Optimizing pain control through the use of implantable pumps. Med Devices (Auckl) 1:41–47
18. Victorov IV, Prass K, Dirnagl U (2000) Improved selective, simple, and contrast staining of acidophilic neurons with vanadium acid fuchsin. Brain Res Brain Res Protoc 5(2):135–139
19. Kinoshita M et al (2006) Targeted delivery of antibodies through the blood–brain barrier by MRI-guided focused ultrasound. Biochem Biophys Res Commun 340(4):1085–1090
20. Kumar P et al (2007) Transvascular delivery of small interfering RNA to the central nervous system. Nature 448(7149):39–43
21. Pardridge WM (2006) Molecular Trojan horses for blood–brain barrier drug delivery. Curr Opin Pharmacol 6(5):494–500
22. Liu XF et al (2004) The window of opportunity for treatment of focal cerebral ischemic damage with noninvasive intranasal insulin-like growth factor-I in rats. J Stroke Cerebrovasc Dis 13(1):16–23
23. Born J et al (2002) Sniffing neuropeptides: a transnasal approach to the human brain. Nat Neurosci 5(6):514–516
24. Huwyler J, Wu D, Pardridge WM (1996) Brain drug delivery of small molecules using immunoliposomes. Proc Natl Acad Sci U S A 93(24):14164–14169
25. Cecchelli R et al (2007) Modelling of the blood–brain barrier in drug discovery and development. Nat Rev Drug Discov 6(8):650–661
26. Abbott NJ et al (2010) Structure and function of the blood–brain barrier. Neurobiol Dis 37(1):13–25
27. Garcel A, Martel S, Carrupt P, Doelker E, Delie F (2010) In vitro blood brain barrier models as a screening tool for colloidal drug delivery systems and other nanosystems. Int J Biomed Nanosci Nanotechnol 1 (2/3/4):133-163
28. Abbott NJ, Romero IA (1996) Transporting therapeutics across the blood–brain barrier. Mol Med Today 2(3):106–113
29. Abbott NJ, Ronnback L, Hansson E (2006) Astrocyte-endothelial interactions at the blood–brain barrier. Nat Rev Neurosci 7(1):41–53
30. Pardridge WM (2008) Re-engineering biopharmaceuticals for delivery to brain with molecular Trojan horses. Bioconjug Chem 19(7):1327–1338
31. Potschka H (2012) Role of CNS efflux drug transporters in antiepileptic drug delivery: overcoming CNS efflux drug transport. Adv Drug Deliv Rev 64(10):943–952
32. Pardridge WM (1992) Recent developments in peptide drug delivery to the brain. Pharmacol Toxicol 71(1):3–10
33. Dietz GP, Bahr M (2004) Delivery of bioactive molecules into the cell: the Trojan horse approach. Mol Cell Neurosci 27(2):85–131
34. Parsons MW et al (2002) Diffusion- and perfusion-weighted MRI response to thrombolysis in stroke. Ann Neurol 51(1):28–37
35. Le Bihan D (2003) Looking into the functional architecture of the brain with diffusion MRI. Nat Rev Neurosci 4(6):469–480
36. Ogawa S et al (1993) Functional brain mapping by blood oxygenation level-dependent contrast magnetic resonance imaging. A comparison of signal characteristics with a biophysical model. Biophys J 64(3):803–812
37. Mace E et al (2011) Functional ultrasound imaging of the brain. Nat Methods 8(8): 662–664
38. Norman D, Brant-Zawadzki M (1985) Magnetic resonance imaging of the central nervous system. Res Publ Assoc Res Nerv Ment Dis 63:259–269
39. Gilman S (1998) Imaging the brain. First of two parts. N Engl J Med 338(12):812–820
40. Moseley I (1995) Imaging the adult brain. J Neurol Neurosurg Psychiatry 58(1):7–21
41. Strakowski SM et al (1999) Brain magnetic resonance imaging of structural abnormalities in bipolar disorder. Arch Gen Psychiatry 56(3):254–260
42. Tsuruda JS et al (1990) Diffusion-weighted MR imaging of the brain: value of differentiating between extraaxial cysts and epidermoid tumors. AJR Am J Roentgenol 155(5):1059–1065, discussion 1066-8

43. Kircher MF et al (2003) A multimodal nanoparticle for preoperative magnetic resonance imaging and intraoperative optical brain tumor delineation. Cancer Res 63(23):8122–8125
44. Albensi BC et al (2000) Diffusion and high resolution MRI of traumatic brain injury in rats: time course and correlation with histology. Exp Neurol 162(1):61–72
45. Ogawa S et al (1990) Brain magnetic resonance imaging with contrast dependent on blood oxygenation. Proc Natl Acad Sci U S A 87(24):9868–9872
46. Krakow K et al (1999) EEG-triggered functional MRI of interictal epileptiform activity in patients with partial seizures. Brain 122(Pt 9):1679–1688
47. Rovaris M et al (2005) Diffusion MRI in multiple sclerosis. Neurology 65(10):1526–1532
48. Kealey SM, Kim Y, Provenzale JM (2004) Redefinition of multiple sclerosis plaque size using diffusion tensor MRI. AJR Am J Roentgenol 183(2):497–503
49. Klucznik RP et al (1993) Placement of a ferromagnetic intracerebral aneurysm clip in a magnetic field with a fatal outcome. Radiology 187(3):855–856
50. Ramirez O 3rd, Jorgensen JS, Thrall DE (1998) Imaging basilar skull fractures in the horse: a review. Vet Radiol Ultrasound 39(5):391–395
51. Mogbo KI et al (1998) Appropriate imaging in children with skull fractures and suspicion of abuse. Radiology 208(2):521–524
52. Bruce DA et al (1981) Diffuse cerebral swelling following head injuries in children: the syndrome of "malignant brain edema". J Neurosurg 54(2):170–178
53. Hu Q et al (2005) Segmentation of brain from computed tomography head images. Conf Proc IEEE Eng Med Biol Soc 4:3375–3378
54. Barrett JF, Keat N (2004) Artifacts in CT: recognition and avoidance. Radiographics 24(6):1679–1691
55. Bracchi M et al (1993) Superficial siderosis of the CNS: MR diagnosis and clinical findings. AJNR Am J Neuroradiol 14(1):227–236
56. Al Kawi MZ, Bohlega S, Banna M (1991) MRI findings in neuro-Behcet's disease. Neurology 41(3):405–408
57. Parizel PM et al (2001) Intracranial hemorrhage: principles of CT and MRI interpretation. Eur Radiol 11(9):1770–1783
58. Reske SN, Kotzerke J (2001) FDG-PET for clinical use. Results of the 3rd German Interdisciplinary consensus conference, "Onko-PET III", 21 July and 19 September 2000. Eur J Nucl Med 28(11):1707–1723
59. Newberg AB, Alavi A (2005) The role of PET imaging in the management of patients with central nervous system disorders. Radiol Clin North Am 43(1):49–65
60. Pan D et al (1998) Rapid synthesis of a 5 -fluorinated oligodeoxy-nucleotide: A model antisense probe for use in imaging with positron emission tomography (PET). Bioorg Med Chem Lett 8(11):1317–1320
61. Antoch G et al (2004) Accuracy of whole-body dual-modality fluorine-18-2-fluoro-2-deoxy-D-glucose positron emission tomography and computed tomography (FDG-PET/CT) for tumor staging in solid tumors: comparison with CT and PET. J Clin Oncol 22(21):4357–4368
62. Ryu JS et al (2002) FDG-PET in staging and restaging non-small cell lung cancer after neoadjuvant chemoradiotherapy: correlation with histopathology. Lung Cancer 35(2):179–187
63. Mosconi L et al (2009) FDG-PET changes in brain glucose metabolism from normal cognition to pathologically verified Alzheimer's disease. Eur J Nucl Med Mol Imaging 36(5):811–822
64. Kumlien E (1998) [Diagnosis of epilepsy by PET scan]. Nord Med 113(2):46–47
65. Kameyama M et al (1990) The accumulation of 11C-methionine in cerebral glioma patients studied with PET. Acta Neurochir (Wien) 104(1–2):8–12
66. Ogawa T et al (1993) Cerebral glioma: evaluation with methionine PET. Radiology 186(1):45–53
67. Harisankar CN et al (2011) FDG-PET findings in fronto-temporal dementia: a case report and review of literature. Indian J Nucl Med 26(2):96–98
68. Bohnen NI et al (2012) Effectiveness and safety of 18F-FDG PET in the evaluation of dementia: a review of the recent literature. J Nucl Med 53(1):59–71

69. Quigley H, Colloby SJ, O'Brien JT (2011) PET imaging of brain amyloid in dementia: a review. Int J Geriatr Psychiatry 26(10):991–999
70. Hartmann A et al (2007) Diagnostic value of 18F-FDG PET/CT in trauma patients with suspected chronic osteomyelitis. Eur J Nucl Med Mol Imaging 34(5):704–714
71. Mitterhauser M et al (2007) Pre vivo, ex vivo and in vivo evaluations of [68Ga]-EDTMP. Nucl Med Biol 34(4):391–397
72. Maecke H, Hofmann M, Haberkorn U (2005) 68Ga-labeled peptides in tumor imaging. J Nucl Med 46(1 suppl):172S–178S
73. Funk T et al (2006) A multipinhole small animal SPECT system with submillimeter spatial resolution. Med Phys 33(5):1259–1268
74. Banerjee S et al (2001) Evolution of Tc-99m in diagnostic radiopharmaceuticals. Semin Nucl Med 31:260–277
75. Choi JY et al (2000) Brain tumor imaging with 99mTc-tetrofosmin: comparison with 201Tl, 99mTc-MIBI, and 18F-fluorodeoxyglucose. J Neurooncol 46(1):63–70
76. Schuh-Hofer S et al (2006) The use of radiolabelled human serum albumin and SPECT/MRI co-registration to study inflammation in the cavernous sinus of cluster headache patients. Cephalalgia 26(9):1115–1122
77. Kosuda S et al (2003) Combined 201Tl and 67Ga brain SPECT in patients with suspected central nervous system lymphoma or germinoma: clinical and economic value. Ann Nucl Med 17(5):359–367
78. Kung HF et al (1990) In vivo SPECT imaging of CNS D-2 dopamine receptors: initial studies with iodine-123-IBZM in humans. J Nucl Med 31(5):573–579
79. Donta ST, Noto RB, Vento JA (2012) SPECT brain imaging in chronic Lyme disease. Clin Nucl Med 37(9):e219–e222
80. Galanaud D et al (2003) Multimodal magnetic resonance imaging of the central nervous system. Biochimie 85(9):905–914
81. Jones EF et al (2008) Nanoprobes for medical diagnosis: current status of nanotechnology in molecular imaging. Curr Nanosci 4(1):17–29
82. Mariani G et al (2010) A review on the clinical uses of SPECT/CT. Eur J Nucl Med Mol Imaging 37(10):1959–1985
83. Chatziioannou A (2005) Instrumentation for molecular imaging in preclinical research: micro-PET and micro-SPECT. Proc Am Thorac Soc 2:533–536
84. Judenhofer MS et al (2008) Simultaneous PET-MRI: a new approach for functional and morphological imaging. Nat Med 14(4):459–465
85. Pichler BJ et al (2010) PET/MRI: paving the way for the next generation of clinical multimodality imaging applications. J Nucl Med 51(3):333–336
86. Goetz C et al (2008) SPECT low-field MRI system for small-animal imaging. J Nucl Med 49(1):88–93
87. Jobsis FF (1977) Non-invasive, infra-red monitoring of cerebral O2 sufficiency, bloodvolume, HbO2-Hb shifts and bloodflow. Acta Neurol Scand Suppl 64:452–453
88. Du C, Pan Y (2011) Optical detection of brain function: simultaneous imaging of cerebral vascular response, tissue metabolism, and cellular activity in vivo. Rev Neurosci 22(6): 695–709
89. Koffie RM et al (2011) Nanoparticles enhance brain delivery of blood–brain barrier-impermeable probes for in vivo optical and magnetic resonance imaging. Proc Natl Acad Sci U S A 108(46):18837–18842
90. Abulrob A et al (2011) In vivo optical imaging of ischemic blood–brain barrier disruption. Methods Mol Biol 763:423–439
91. Jia Y et al (2011) In vivo optical imaging of revascularization after brain trauma in mice. Microvasc Res 81(1):73–80
92. Hillman EM (2007) Optical brain imaging in vivo: techniques and applications from animal to man. J Biomed Opt 12(5):051402
93. Trachtenberg JT et al (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420(6917):788–794

94. Vasquez KO, Casavant C, Peterson JD (2011) Quantitative whole body biodistribution of fluorescent-labeled agents by non-invasive tomographic imaging. PLoS One 6(6):e20594
95. Tosi G et al (2011) NIR-labeled nanoparticles engineered for brain targeting: in vivo optical imaging application and fluorescent microscopy evidences. J Neural Transm 118(1):145–153
96. Hebden JC et al (2002) Three-dimensional optical tomography of the premature infant brain. Phys Med Biol 47(23):4155–4166
97. Franceschini MA et al (2007) Assessment of infant brain development with frequency-domain near-infrared spectroscopy. Pediatr Res 61(5 Pt 1):546–551
98. Cannestra AF et al (2004) Functional magnetic resonance imaging and optical imaging for dominant-hemisphere perisylvian arteriovenous malformations. Neurosurgery 55(4):804–812, discussion 812–4
99. D'Arceuil HE et al (2005) Near-infrared frequency-domain optical spectroscopy and magnetic resonance imaging: a combined approach to studying cerebral maturation in neonatal rabbits. J Biomed Opt 10(1):11011
100. Slovin H et al (2002) Long-term voltage-sensitive dye imaging reveals cortical dynamics in behaving monkeys. J Neurophysiol 88(6):3421–3438
101. Kircher MF et al (2012) A brain tumor molecular imaging strategy using a new triple-modality MRI-photoacoustic-Raman nanoparticle. Nat Med 18(5):829–834
102. Fan CH et al (2013) SPIO-conjugated, doxorubicin-loaded microbubbles for concurrent MRI and focused-ultrasound enhanced brain-tumor drug delivery. Biomaterials 34(14):3706–3715
103. Wadghiri YZ et al (2013) Detection of amyloid plaques targeted by bifunctional USPIO in Alzheimer's disease transgenic mice using magnetic resonance microimaging. PLoS One 8(2):e57097
104. Chen CCV, Shih YYI, Chang C (2013) Dopaminergic imaging of nonmotor manifestations in a rat model of Parkinson's disease by fMRI. Neurobiol Dis 49(1):99–106
105. Venneti S, Lopresti BJ, Wiley CA (2013) Molecular imaging of microglia/macrophages in the brain. Glia 61(1):10–23
106. Gupta RK et al (2012) Imaging of central nervous system viral diseases. J Magn Reson Imaging 35(3):477–491
107. Guzman R et al (2007) Long-term monitoring of transplanted human neural stem cells in developmental and pathological contexts with MRI. Proc Natl Acad Sci U S A 104(24):10211–10216
108. Bulte JWM (2009) In vivo MRI cell tracking: clinical studies. Am J Roentgenol 193(2):314–325
109. Li Calzi S et al (2009) Labeling of stem cells with monocrystalline iron oxide for tracking and localization by magnetic resonance imaging. Microvasc Res 78(1):132–139
110. Voura EB et al (2004) Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. Nat Med 10(9):993–998
111. Bentolila LA, Ebenstein Y, Weiss S (2009) Quantum dots for in vivo small-animal imaging. J Nucl Med 50(4):493–496
112. Adonai N et al (2002) Ex vivo cell labeling with 64Cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) for imaging cell trafficking in mice with positron-emission tomography. Proc Natl Acad Sci U S A 99(5):3030–3035
113. Cromer Berman SM, Walczak P, Bulte JW (2011) Tracking stem cells using magnetic nanoparticles. Wiley Interdiscip Rev Nanomed Nanobiotechnol 3(4):343–355
114. Wang FH et al (2006) Magnetic resonance tracking of nanoparticle labelled neural stem cells in a rat's spinal cord. Nanotechnology 17(8):1911–1915
115. Yi P et al (2013) Magnetic resonance imaging of Fe3O4@SiO2-labeled human mesenchymal stem cells in mice at 11.7T. Biomaterials 34(12):3010–3019
116. Huang X et al (2013) Mesenchymal stem cell-based cell engineering with multifunctional mesoporous silica nanoparticles for tumor delivery. Biomaterials 34(7):1772–1780
117. Lonser RR et al (2007) Image-guided, direct convective delivery of glucocerebrosidase for neuronopathic Gaucher disease. Neurology 68(4):254–261

118. Langer R (1990) New methods of drug delivery. Science 249(4976):1527–1533
119. Pardridge WM (1997) Drug delivery to the brain. J Cereb Blood Flow Metab 17(7):713–731
120. Bidros DS, Liu JK, Vogelbaum MA (2010) Future of convection-enhanced delivery in the treatment of brain tumors. Future Oncol 6(1):117–125
121. Chen MY et al (1999) Variables affecting convection-enhanced delivery to the striatum: a systematic examination of rate of infusion, cannula size, infusate concentration, and tissue-cannula sealing time. J Neurosurg 90(2):315–320
122. Sawyer AJ et al (2011) Convection-enhanced delivery of camptothecin-loaded polymer nanoparticles for treatment of intracranial tumors. Drug Deliv Transl Res 1(1):34–42
123. Sawyer AJ, Piepmeier JM, Saltzman WM (2006) New methods for direct delivery of chemotherapy for treating brain tumors. Yale J Biol Med 79(3–4):141–152
124. Hynynen K (2007) Focused ultrasound for blood–brain disruption and delivery of therapeutic molecules into the brain. Expert Opin Drug Deliv 4(1):27–35
125. Patrick JT et al (1990) Ultrasound and the blood–brain barrier. Adv Exp Med Biol 267:369–381
126. McDannold N et al (2004) MRI investigation of the threshold for thermally induced blood–brain barrier disruption and brain tissue damage in the rabbit brain. Magn Reson Med 51(5):913–923
127. Hynynen K et al (2001) Noninvasive MR imaging-guided focal opening of the blood–brain barrier in rabbits. Radiology 220(3):640–646
128. Choi JJ et al (2007) Noninvasive, transcranial and localized opening of the blood–brain barrier using focused ultrasound in mice. Ultrasound Med Biol 33(1):95–104
129. Wong HL et al (2007) Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles. Adv Drug Deliv Rev 59(6):491–504
130. Fortin D et al (2007) Enhanced chemotherapy delivery by intraarterial infusion and blood–brain barrier disruption in the treatment of cerebral metastasis. Cancer 109(4):751–760
131. Wong HL, Wu XY, Bendayan R (2012) Nanotechnological advances for the delivery of CNS therapeutics. Adv Drug Deliv Rev 64(7):686–700
132. Allhenn D, Boushehri MA, Lamprecht A (2012) Drug delivery strategies for the treatment of malignant gliomas. Int J Pharm 436(1–2):299–310
133. Moghimi SM, Hunter AC, Murray JC (2005) Nanomedicine: current status and future prospects. FASEB J 19(3):311–330
134. van Rooy I et al (2011) In vivo methods to study uptake of nanoparticles into the brain. Pharm Res 28(3):456–471
135. Hadjipanayis CG et al (2010) EGFRvIII antibody-conjugated iron oxide nanoparticles for magnetic resonance imaging-guided convection-enhanced delivery and targeted therapy of glioblastoma. Cancer Res 70(15):6303–6312
136. Biddlestone-Thorpe L et al (2012) Nanomaterial-mediated CNS delivery of diagnostic and therapeutic agents. Adv Drug Deliv Rev 64(7):605–613

Chapter 5
Inhalation Therapy for Pulmonary Tuberculosis

Keiji Hirota, Keishiro Tomoda, Kimiko Makino, and Hiroshi Terada

5.1 Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is one of the worst of infectious diseases. *Mtb* is transmitted from person to person via airborne droplets that arise from the one infected with the active *Mtb* organism, and thus this mode of transmission has the potential to cause a worldwide pandemic. In addition, the recent emergence of multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) has become a major public health issue in both developing and developed countries. Although the development of new anti-TB drugs, such as TMC207, OPC-67683, and PA-824, is recently in progress throughout the world [1, 2], the establishment of an effective system for the delivery of such drugs as well as conventional anti-TB drugs also needed for the extermination of TB. In this chapter, essential medical treatment and promising formulations for the treatment of pulmonary TB through inhalation are described.

K. Hirota • K. Tomoda • K. Makino
Center for Drug Delivery Research, Faculty of Pharmaceutical Sciences, and Center for Physical Pharmaceutics, Research Institute for Science and Technology, Tokyo University of Science, Yamazaki, Noda, Chiba, Japan

H. Terada (✉)
Center for Drug Delivery Research, Faculty of Pharmaceutical Sciences, and Center for Physical Pharmaceutics, Research Institute for Science and Technology, Tokyo University of Science, Yamazaki, Noda, Chiba, Japan

Niigata University of Pharmacy and Applied Life Sciences, 265-1, Higashijima, Akiha-ku, Niigata City 956-8603, Japan
e-mail: teradah@rs.tus.ac.jp

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_5

5.1.1 Epidemiology of TB

The Global Tuberculosis Report 2012, published by World Health Organization (WHO), stated that "the WHO declared TB a global public health emergency in 1993. Starting in the mid-1990s, efforts to improve TB care and control intensified at national and international levels" [3]. However, one-third of the world population has already been infected with *Mtb* [4, 5]; and the latest estimates by the WHO showed that 8.7 million people fell ill with TB, and 1.4 million people died from it, in 2011 [3].

In addition, the risk of TB pathogenesis in people infected with the human immunodeficiency virus (HIV) is 21–34 times greater than that for those without HIV infection [6]; and TB is the leading cause of death among HIV-positive individuals, being responsible for 22 % of HIV-related deaths. As people infected with both HIV and *Mtb* become a growing pool of the *Mtb*, treatment and prevention of TB need to be addressed in conjunction with acquired immunodeficiency syndrome (AIDS).

5.1.2 Pathology of TB

Mtb organisms infect the lung tissue of an individual through inhalation of the *Mtb-containing* airborne droplets that arise from coughing and conversing with TB patients. The initial infection with *Mtb* has a potential to occur in any of the lung lobes, which infection leads to the formation of small necrotic caseous lesions that contain *Mtb*s engulfed by macrophages and other recruited leukocytes. The onset of recognizable TB disease following the initial infection is evident in only a few individuals. In general, TB turns active and infectious when the bacteria have developed a secondary site of infection, which is usually the upper lobes of the lungs, though the mechanism remains unknown.

Alveolar macrophages patrol the alveoli and eliminate exogenous foreign substances by endocytic ingestion and subsequent exposure to reactive oxygen species (ROS) and digestive enzymes. As shown in Fig. 5.1, pathogens are first taken into

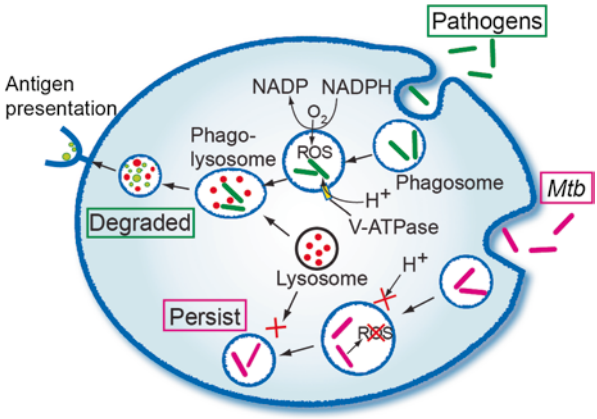

Fig. 5.1 Survival of *Mtb* in alveolar macrophage

phagosomes in macrophages through phagocytic pathways. Then, in general the pathogens are exposed to ROS generated via NADPH oxidase and to lysosomal enzymes after the fusion of the phagosomes with lysosomes to form phagolysosomes; and finally they undergo full digestion. However, *Mtb* organisms can escape from the clearance process by inhibiting this fusion of the phagosomes with the lysosomes by blocking V-ATPase expression and increasing the accumulation of TACO (tryptophan-aspartate-containing coat protein) around the phagosome membrane [7–9]. In addition, *Mtbs* become resistant to attack by ROS in the macrophages by generating catalases, peroxidases, and superoxide dismutases [10, 11]. These mechanisms allow *Mtbs* to persist in the macrophages.

The macrophages infected with *Mtbs* subsequently generate inflammatory responses that lead to the recruitment of neutrophils, monocytes, other macrophages, T cells, and B cells from neighboring blood vessels and lymph nodes. Recruited macrophages are transformed into several cell types such as epithelioid cells and Langhans giant cells (generated by fusion of epithelioid cells) during the prolonged inflammatory response, and these cells and the recruited lymphocytes form a granuloma that encloses the bacteria, a process taking approx. 2 years [12–14]. However, the *Mtbs* exploit this granuloma as a shelter to escape from monitoring by immune cells and attacks by antibiotics and thus persist in the lungs.

Mtb grows in the lungs by utilizing host-derived lipids as a carbon source [15, 16]. During the growth, caseation and cavitation gradually progress at the core of the granuloma. Then, the organisms that have proliferated exploit macrophages as well as neutrophils to be carried as a cargo and to move into the upper airways with the sputum [17]. The presence of *Mtbs* in the sputum indicates the infectious state. As *Mtb* latently infects individuals, a relapse of TB is associated with a depression of immune activity due to aging, a surgical operation, anti-TNF-α therapy or HIV infection.

5.2 TB Therapy

5.2.1 Current Treatment

Current treatment of TB is performed by oral intake of multiple anti-TB drugs according to the internationally accepted first-line treatment regimen [18]. The drugs used in the first-line treatment are isoniazid (INH), rifampicin (RFP), pyrazinamide (PZA), and ethambutol (EB). Sometimes streptomycin (SM) is employed instead of EB. These therapeutic drugs, except EB, possess mycobactericidal activities. The second-line anti-TB drugs include aminoglycosides, such as amikacin (AMK) and kanamycin (KM); capreomycin (CPM); fluoroquinolones; and *p*-aminosalicylic acid (PAS). These drugs are mainly employed in the treatment of MDR/XDR-TB due to their weaker anti-TB activities and greater toxicity than those used in the first-line treatment.

The standard regimen is effective to kill 99 % of TB pathogens within 1 month, but elimination of the remaining 1 % takes 3 months [19]. In addition, an additional

dose for 2 months after the disappearance of the bacterial burden in the lungs is needed to prevent a relapse of TB. The current therapy thus has a long duration of more than 6 months for a complete recovery from TB [19].

However, the continuous long-term dosing often ends in failure in TB treatment and consequently causes the emergence of drug-resistant organisms. To prevent the failure of TB therapy, the WHO has proposed the treatment of directly observed therapy, short course (DOTS), which has contributed to high drug compliance. Although the introduction of DOTS has resulted in increases in successful treatment outcomes [20], novel anti-TB drugs acting based on mechanisms other than those by conventional anti-TB drugs, and optimum therapeutic protocols to eliminate *Mtb* over the short term of less than 6 months, are needed for complete treatment of TB. In addition, overcoming MDR/XDR-TB is also a major clinical issue to be addressed.

5.2.2 Inhalation TB Therapy

The *Mtb* that has invaded into the lungs easily infects alveolar macrophages, in which it proliferates by inhibiting phagosome-lysosome fusion by several mechanisms previously described in Sect. 1.2. Hence, the infected alveolar macrophages should be a primal target for TB therapy, because the organisms accumulate in these cells [21]. Comparison between inhalation therapy and conventional oral dosing for TB treatment is summarized in Table 5.1.

The amount of inhaled anti-TB drug in alveolar macrophages is expected to be greater than that administered by oral dosing, if an inhalable formulation that incorporates an anti-TB drug and is easily taken up by alveolar macrophages is available. It is reported that hydrophilic aminoglycosides in a form of an aerosol can sterilize *Mtbs* in human sputum and reduce the burden of *Mtb* in the mouse lung [22, 23]. In addition, anti-TB drugs formulated into microparticles having a diameter of 3 μm, which is suitable for ingestion by alveolar macrophages [24], should contribute to their efficient deposition in the lungs. The delivery of anti-TB drugs directly into the lungs also decreases their concentration in the circulation system and thus avoids adverse effects such as hepatic dysfunction and neurologic disorders. Hence, inhalation therapy is promising for complete treatment of TB.

Table 5.1 Comparisons between inhalation therapy and oral treatment for TB

	Inhalation therapy	Oral treatment
Drug distribution	Local	Systemic
Dose	Milligram order	Gram order
Medication	Inhalation using special devices	Intake with water
Formulations	Liquid, suspension, dry powder	Tablet, capsule
Adverse effects	Unknown, but expected to be nontoxic	Hepatic dysfunction, neurologic disorders, allergic response

5.3 Preparation of Inhalable Particles Containing Anti-TB Drugs

The human airway becomes narrower after the bifurcation forming the bronchi, decreasing in diameter from 1.8 cm at the entry of the trachea to 0.041 cm at the distal airway [25]. As the alveolar macrophages reside in the deepest region of the lungs, there is a size limitation of the particles reaching there. In addition, an effective particle size in the airflow is different from its geometric size due to the emergence of a drag force dependent on the velocity; and this effective size is defined as the aerodynamic diameter (D_{aer}), as shown in the equation below:

$$D_{aer} = D_{geo} \times \sqrt{\frac{\rho}{\chi}},$$

where D_{geo} is the geometric diameter, ρ is the particle density, χ is the shape factor (a sphere gives 1; but elongated particles, such as fibers and needles, are greater than 1) [26]. This aerodynamic diameter is useful to estimate where particles will become deposited in the respiratory system. From the mathematical model based on the experimental data, it has been shown that particles with a D_{aer} between 1 and 5 μm are efficiently deposited in the alveolar pulmonary region [27]. Most of the larger particles of D_{aer} >10 μm are trapped at the oropharynx, whereas smaller particles of D_{aer} <1 μm reach the alveoli; however, most of them are exhaled without settlement in the lungs [28]. Methods for the preparation of inhalable particles for TB therapy are summarized in the following sections:

5.3.1 Emulsion/Solvent Evaporation

There are a considerable number of reports on the formulation of microparticles and nanoparticles of various biomaterials for the delivery of anti-TB drugs. Biodegradable materials are advantageous for the formulation of particles used for long-lasting drug release [29]. One of the most frequently employed methods to formulate the particles is based on the emulsion/solvent evaporation method. The emulsification is conducted by mixing a dispersed oil phase into a continuous aqueous phase. For preparation of the particles, hydrophobic polymers, such as poly(lactic-co-glycolic) acid (PLGA) as bases, and hydrophobic anti-TB drugs, such as RFP, as active ingredients, are dissolved in an organic solvent such as dichloromethane and emulsified in an aqueous solution, in which both the base polymer and the drug are insoluble in the presence of emulsifiers, such as glycerol, poly vinyl alcohol and surfactants, under continuous agitation [30, 31]. The dispersed particles that have encapsulated the drugs are obtained after evaporation of the organic solvent. The hydrophilic INH and hydrophobic RFP have been encapsulated into particles successfully obtained by use of suitable solvents [32]. Particle size and its distribution depend on mixing methods, agitation speed, and concentration of the solutes. Extrusion of the

dispersed phase into the aqueous phase through a Shirasu porous glass membrane under pressurization using inactive N_2 gas allows the formulation of particles with a narrow size distribution [33]. The main limitation of the emulsion/solvent evaporation method is the small batch size for preparation of the particles.

5.3.2 Spray-Drying

Spray-drying is one of the most commonly employed techniques to formulate dry powders in sizes from nanometers through micrometers. In general, active pharmaceutical ingredients (API) and excipients are dissolved in an aqueous or organic solvent, and the liquid is sprayed through a narrow atomization nozzle with high pressurized inactive gas at a temperature higher than the vaporization point of the solvent. The fine droplets emitted are quickly dried, and then the generated particles are collected in a cyclone chamber or an electrostatic precipitator. The dry powders formulated by the spray-drying method contain an accurate amount of API and excipients [30, 34]. It is noteworthy that the spray-drying method is available to formulate proteinaceous APIs into particles. Exubera, known as the first commercial proteinaceous inhalable dry microparticle product but now withdrawn from the market, which consisted of recombinant human insulin, as an API, and mannitol, glycine, and sodium citrate, as the excipients, was produced by the spray-drying method [35]. The spray-drying method is more advantageous than the emulsion/solvent evaporation one for the preparation of a large amount of stable particles at the industrial level.

A number of inhalable particles containing anti-TB drugs have been prepared by spray-drying. RFP was formulated in a form of inhalable dry particles with a carrier material of PLGA by the spray-drying method [36–39], and it was found that the release period of RFP from the particles increased with an increase in the molecular weight and ratio of lactic acid to glycolic acid in the PLGA. Dry powders containing PA-824, an anti-TB drug under development or multiple drugs such as hydrophilic INH and hydrophobic RFP could also be prepared by the spray-drying method [40, 41]. For the treatment of MDR/XDR-TB, capreomycin was spray-dried into an inhalable formulation; and a phase I clinical trial of the formulation was completed [42, 43]. In addition, it is noteworthy that the spray-drying approach allowed for preparing dry powders containing viable vaccine strain Bacillus Calmette–Guérin (BCG) without freezing [44].

Spray-freeze-drying, similar to the spray-drying, consists of the combination of spray-drying and freeze-drying methods; and it is useful for the preparation of dry powders, especially for those to be used with heat-sensitive molecules such as proteins and peptides without causing a decrease in their activities [45, 46]. The spraying is performed into a cryogenic liquid such as liquid nitrogen, and the drying is conducted at ambient temperature. This method is efficient for the preparation of lighter and more porous dry powders than those obtained by spray-drying, with a high yield of almost 100 %.

5.3.3 *Liposomes*

The delivery of drugs directly to the lungs by nebulization has been attempted by the use of aerosolized liposome suspensions. Lipids, such as phospholipids, are major components of mammalian organisms; and hence they are regarded as biocompatible, and the aqueous space inside them is favorable for trapping hydrophilic drugs. Liposomes containing AMK, an aminoglycoside antibiotic, known as Arikace, are being used for inhalation treatment of gram-negative infections [47]. Arikace is currently proceeding under a phase II clinical study.

The specific targeting of liposomes toward nests of *Mtb*, namely, infected alveolar macrophages, was achieved by modifying the surface of the liposomes with maleylated bovine serum albumin (MBSA) and *O*-steroyl amylopectin (*O*-SAP) [48]. These macrophage-specific ligands increased the delivery of anti-TB drugs encapsulated by the liposomes into the macrophages, which delivery was followed by efficient attacking of the *Mtbs* residing in the intracellular space. Liposomal formulations are likely to progress to routine clinical usage within a short period compared with the polymer particles due to their biocompatible safety, as was shown in pediatric use such as treatment of neonatal respiratory distress syndrome [49]. However, several problems, such as a low encapsulation rate of certain polar drugs, high burst release of drugs, and a short shelf-life, need to be overcome for their practical application [50].

5.4 Effect of Inhaled Particles on Macrophage Functions

As mentioned above, macrophages infected with *Mtbs* should be an effective target for the delivery of anti-TB drugs [21]. Particle formulation is considered to be important for the import of anti-TB drugs into macrophages and is based on the high endocytic activity of macrophages. Endocytic uptake of bacteria and exogenous particles is regarded to be mainly dependent on their sizes and vesicle formation upon ingestion [51–54]. Phagocytosis, one of the ingestion routes, covers the uptake of particles having a diameter of more than 0.5 μm; macropinocytosis, those with one from 0.1 through 5 μm; and pinocytosis, those less than 120 nm in diameter [55–57]. The optimum size for attaining efficient uptake of anti-TB drugs by alveolar macrophages was reported to be around 3 μm [24].

It is noteworthy that inflammatory responses are triggered by the uptake of particles from macrophages. The signals generated during such a response could represent cautious signals to notify the surrounding immune cells of an attack by invaders. These signals are initiated by pattern recognition receptors (PRRs), which identify pathogen-associated molecular patterns (PAMPs) [58]. Hence, contamination by PRR ligands including components of bacterial cell walls, such as lipopolysaccharide and lipoarabinomannan, should be avoided when determining the exact mechanism of interaction between inhaled particles and macrophages.

Nanoparticles associated with polyethylene glycol (PEG) are known as a stealth formulation and are favorable to allow these particles to avoid interacting with immune cells. This formulation is practically utilized for the liposomal formulation of doxorubicin DOXIL (doxorubicin HCl liposome injection) used for the treatment of ovarian cancer and Kaposi's sarcoma [59]. However, the utilization of PEG to obtain these stealth particles caused unintentional production of anti-PEG IgM antibody from splenic B cells, and the subsequent clearance of the particles was consequently accelerated [60–62]. The silent stealth nature and concealment, as in the case of a "Ninja," is a key consideration for the development of formulations for inhalation delivery [63, 64].

5.5 Animal Studies

It is very difficult to deliver particles to the lungs homogenously by inhalation. Delivery is relatively well achieved for humans and large animals having a lung size similar to the human one. However, the delivery to small animals, such as mice, rats, and guinea pigs, is much more difficult, because the lung size of these animals is obviously very small. In the case of mice, the air space of the lungs has a volume of approx. 1 ml; and the internal diameter of the upper trachea is about 1 mm. In order to evaluate efficacies of inhalation formulations in these small animals, whole-body, head-only, and nose-only exposure inhalation chambers have been exploited [65–67]. However, it is difficult to determine quantitatively the amount of drugs delivered into the lung tissue by use of exposure inhalation chambers due to the deposition of the drugs in the unintended tissues, such as the nasal cavity and skin. In addition, the use of such chambers requires a massive amount of dry powders, which is disadvantageous in terms of the handling and cost of dry powders. Consequently, intratracheal administration, such as instillation, nebulization, and insufflation, has been employed to deliver drugs to the lungs of small rodent animals. Characteristics of various methods for drug delivery to the lungs of laboratory animals are summarized in Table 5.2. In the next section, we will review noninvasive methods for drug delivery to the lungs.

5.5.1 Inhalation Administration of Drugs into the Lungs

5.5.1.1 Intratracheal Administration

Accurate delivery of drugs to the lungs is considered to be achievable by intratracheal administration. For this goal, an endotracheal cannula of a suitable size should be prepared for intubation; and a careful examination of the pharynx and larynx is necessary. The internal diameter of the trachea of laboratory rodents is approx. 1–3 mm. Prior to intubation, the animal is anesthetized to a sufficient depth to abolish

Table 5.2 Methods for drug delivery to the lungs

	Nose-only exposure chamber	Intratracheal administration		
		Insufflation	Nebulization	Instillation
Animals	Mice, rats, guinea pigs			
Required dose	Gram order	Milligram order	Microgram to milligram order	Microgram to milligram order
Formulations	Liquid, suspension, dry powder	Dry powder	Liquid, suspension	Liquid, suspension
Synchronization with breath	Yes	No	Yes/no	No
Distribution in the lungs	Homogeneous	Heterogeneous	Heterogeneous	Heterogeneous
Quantitative dosing	No	Yes	Yes	Yes
Repeated dosing	Yes	Yes/no	Yes/no	Yes/no
Delivery to unintended region	Nasal cavity, throat, skin	None	None	None
Anesthesia	Dispensable	Necessary	Necessary	Necessary
Batch size	A large number of animals	A single animal	A single animal	A single animal
Cost	Very high	Low	Low-high	Very low

the cough and swallowing reflexes. The use of anesthetic agents that cause no or only weak irritation of the respiratory tract is important for intubation. The inhalation anesthetics halothane and isoflurane, and the intravenous anesthetic propofol are likely to be favorable to avoid the over-secretion of saliva and airway mucus. In addition, anticholinergic agents are useful for reducing the mucus secretion and preventing tube blockage. Visualization of the entrance of the trachea is also important to perform the intubation properly and may be achieved by using a laryngoscope or an otoscope [68, 69]. Alternatively, illumination of the neck with a powerful light source helps visualization of the oropharynx [70].

Administration of liquid and suspended materials is carried out by intratracheal instillation. The volume for instillation is an important factor to achieve wide spreading of the drug in the lung tissue. Namely, 1–2 ml/kg body weight seems to be a favorable one. It is noteworthy that the amount of test drugs in the liquid dosing is restricted by their solubility in the limited volume of the liquid medium. In general, a slight amount of less than 1 mg may be favorable. Viscous liquid samples can be delivered into the lungs by intratracheal instillation, but the possible occurrence of respiratory failure should be taken into account.

Intratracheal nebulization is also useful to deliver low viscous liquid and suspended drugs into the lungs, and making use of a specialized apparatus, such as the MicroSprayer Aerosolizer Model IA–1C with FMJ-250 High Pressure Syringe (Penn-Century, Inc., Philadelphia, USA), is effective. The droplet size of aerosolized

phosphate-buffered saline obtained with the MicroSprayer was reported to be around 8 μm in terms of mean mass aerodynamic diameter [70]. Other specialized apparatuses, such as the nebulization catheter AeroProbe (Trudell Medical International, London, Canada), allow for pulmonary aerosol administration with a breath-controlled dosing. About 12 % of the aerosol dosed by use of this catheter device was reported to be delivered to the rat lung parenchyma [71]. However, the aerosols delivered into the lungs were mainly trapped at tracheobronchial sites owing to large mass median diameter of the droplets, which was 11 μm [71]. It is important to design nebulizers targeting deep into the lung in such a way that they administer aerosol particles having aerodynamic diameters between 1 and 5 μm, which is the optimal aerodynamic size for delivering drugs deep into the lung tissue for efficient intratracheal delivery [27].

Models of Penn-Century type intratracheal insufflator are commonly employed for pulmonary administration of dry powders. The standard chamber of the device holds dry powders up to 5 mg, and the insufflation of the powders is executed by forcing air through an actuating syringe. Air pressure and volume must be adjusted to accommodate well the respiration of small rodents having a tidal volume of less than 2 ml. In general, the insufflation of a small amount of dry powder, less than 1 mg, is difficult.

5.5.1.2 Exposure Inhalation Chambers

There are numerous reports on exposure inhalation chambers, such as whole-body, head-only, and nose-only types [65, 66]. These chambers are equipped with various types of airflow generation systems. Of these, the flow-past chamber is favorable for exposing all test animals with the same aerosol concentration to attain uniform delivery of drugs and for reducing the amount of drugs expended [72]. In general, a dust generator, a nebulizer, or a metered dose inhaler is equipped as an aerosol generator. Particle sizes and amount of inhaled particles in the test animals can be estimated from the analysis of particles in the chamber atmosphere. However, the exposure inhalation chamber system is much more expensive than intratracheal administration apparatuses. Alternatively, hand-crafted inhalation chambers based on a plastic centrifuge tube have been utilized for dry powder administration to mice and rats [32, 73].

One of the major problems in using an inhalation exposure chamber is that the actual amount of test materials deposited in the lungs cannot be determined accurately. In general, the received dose is estimated by the following equation:

$$D = C \times V_{\mathrm{m}} \times t \times \mathrm{Fr},$$

where D = dose, C = concentration of test sample, V_{m} = volume for the minute ventilation, t = exposure duration, Fr = fraction of test sample deposited or absorbed [74]. Alternatively, the dose deposited in the lungs can be determined by measuring the plasma concentration of the inhaled agent [75].

5.5.2 Pulmonary Deposition of Inhaled Particles

Evaluation of pulmonary deposition of inhaled particles provides important information regarding drug action. The amount of particles in the lungs is determined from that in the bronchoalveolar lavage fluid and in the lung homogenate of the test animals. In general, the amount of drugs deposited in the lungs by inhalation increases more rapidly with time than that administered by intravenous injection. As a result, the total amount in the lungs is greater than that by intravenous administration. However, the drug administered by inhalation disappears similarly as that by injection within 12 h [76]. The drug in the lungs is eliminated mainly by the pulmonary defense system, such as mucociliary clearance toward the upper airways and by transference into the blood. Hence, it is necessary to construct a strategy to minimize such a rapid disappearance of the deposited drug for achievement of the long-lasting effect of inhaled particles in the lung tissue.

Inhaled particles deposited in the lungs can be detected microscopically in lung sections by use of particles labeled with fluorescent probes. Fresh lung tissue is sliced to a thickness of approx. 2 mm without being embedded in paraffin and is then observed under a confocal laser scanning microscope [77]. In addition, it is worth noting that the Kawamoto method enables the preparation of whole-lung sections with a thickness of 10 μm from frozen lungs [78]. The distribution of inhaled particles in the lungs was easily determined by using such whole-lung sections [79]. In contrast, scintigraphic imaging using radioisotopes such as ^{99m}Tc is favorable for real-time monitoring of drug deposition in lung tissue in 2D or 3D without sacrificing the test animals [80]. The radiolabeling of the drug with ^{99m}Tc is conducted by mixing the two in a solvent in which the drug is insoluble but ^{99m}Tc is soluble, such as water, followed by drying of the mixture [81]. Such a labeling procedure is of importance for tracking the pulmonary deposition of drugs time-dependently and accurately.

5.5.3 Anti-TB Activity of Inhaled Particles

The efficacy of inhaled anti-TB drugs is evaluated by measuring the anti-TB activity in terms of a decrease in the number of colony-forming units (CFU) in TB animal models. As summarized in Table 5.3, various anti-TB drugs, along with various dose regimens, have been used to treat animal models of TB [37, 75, 82–85]. Among them, the guinea pig is favorable as a model animal in TB studies; because its pathogenesis in terms of the formation of granulomas and caseous necrosis regions is similar to that seen in human TB [86].

As shown in Table 5.3, a significant decrease in mycobacterial burden in the lungs seemed not to be achieved in most of the cases; even though a large amount of anti-TB drugs was delivered in a form of an inhalation formulation to the lungs [37, 75, 82–84]. The lesser effectiveness of the inhaled anti-TB drugs than expected

Table 5.3 Drugs formulated for inhalation treatment of TB and their experimental results

Drug	Carrier	Dose and repeat times	Delivery method	Animal	TB model	Drug concentration	CFU[a]	Ref
Rifampicin	PLGA	12 mg/kg and 5 mg/kg Each once	Insufflation and nebulization	Guinea pig	*Mtb* H37Rv, subacute	N.D.[b]	0.71	[37]
Rifampicin	PLGA	540 mg/kg Four times	Nebulization	Guinea pig	*Mtb* H37Rv, subacute	N.D.	N.E.[c]	[82]
Rifampicin	PLGA	300 μg/kg 14 times	Insufflation	Rat	*Mtb* Kurono, acute	N.D.	0.23	[83]
Capreomycin	Leucine	1.80 mg/kg[d] 28 times	Insufflation and inhalation	Guinea pig	*Mtb* H37Rv, chronic	C_{max}: 6.70 ± 1.26 μg/mL in plasma (at 19 min)	1.03	[84]
PA-824	Leucine, DPPC	9.7 mg/kg[d] 28 times	Inhalation	Guinea pig	*Mtb* H37Rv, chronic	C_{max}: 0.59 μg/mL in plasma (at 3 h)	0.80	[75]
Clofazimine	Leucine	28.8 mg/kg Eight times	Inhalation	Mouse	*Mtb* H37Rv, chronic	N.D.	2.61	[85]

[a]Decrease in CFU from that with no treatment (log scale)
[b]N.D. not determined
[c]N.E. not effective
[d]Calculated from plasma concentration

might have been because the inhaled anti-TB drugs had not been delivered deep enough into the lungs, where *Mtb* resides. Hence, it is necessary to develop devices that enable the delivery of an effective amount of anti-TB drug to the tuberculosis granulomatous nests, which reside deep in the lungs, as well as to prepare more efficient inhalable formulations of anti-TB drugs. Of the inhalation drugs under development, capreomycin dry powders have successfully proceeded to a phase I clinical study [43].

5.6 Conclusions

Mtbs that have invaded the lungs during respiration are easily taken up by alveolar macrophages. Hence, delivery of anti-TB drugs directly to the alveolar macrophages that have been infected with this organism is expected to be more effective in terms of anti-TB activity than their oral administration. However, such an inhalation therapy for TB has not yet been established and remains in the development stage. In order for inhaled anti-TB drugs to exert potent mycobactericidal activity, the development of inhalable formulations containing anti-TB drugs and that of devices that achieve efficient delivery of a curable amount of these formulations to the macrophages are necessary. Inhalation therapy for TB will surely contribute to radical treatment of TB including MDR/XDR TB.

References

1. Gler MT, Skripconoka V, Sanchez-Garavito E, Xiao H, Cabrera-Rivero JL, Vargas-Vasquez DE, Gao M, Awad M, Park SK, Shim TS, Suh GY, Danilovits M, Ogata H, Kurve A, Chang J, Suzuki K, Tupasi T, Koh WJ, Seaworth B, Geiter LJ, Wells CD (2012) Delamanid for multidrug-resistant pulmonary tuberculosis. N Engl J Med 366:2151–2160
2. Tasneen R, Li SY, Peloquin CA, Taylor D, Williams KN, Andries K, Mdluli KE, Nuermberger EL (2011) Sterilizing activity of novel TMC207- and PA-824-containing regimens in a murine model of tuberculosis. Antimicrob Agents Chemother 55:5485–5492
3. Global Tuberculosis Report (2012) World Health Organization. http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf. Accessed 15 Apr 2013
4. Barnes PF, Cave MD (2003) Molecular epidemiology of tuberculosis. N Engl J Med 349: 1149–1156
5. Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C (2003) Tuberculosis. Lancet 362: 887–899
6. HIV/TB Facts (2011) World Health Organization. http://www.who.int/hiv/topics/tb/hiv_tb_factsheet_june_2011.pdf. Accessed 15 Apr 2013
7. Armstrong JA, Hart PD (1971) Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J Exp Med 134:713–740
8. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG (1994) Lack of acidification in mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263:678–681
9. Ferrari G, Langen H, Naito M, Pieters J (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 97:435–447

10. Manca C, Paul S, Barry CE 3rd, Freedman VH, Kaplan G (1999) Mycobacterium tuberculosis catalase and peroxidase activities and resistance to oxidative killing in human monocytes in vitro. Infect Immun 67:74–79
11. Voskuil MI, Bartek IL, Visconti K, Schoolnik GK (2011) The response of mycobacterium tuberculosis to reactive oxygen and nitrogen species. Front Microbiol 2:105
12. Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F (2009) Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol 10:943–948
13. Russell DG, Barry CE 3rd, Flynn JL (2010) Tuberculosis: what we don't know can, and does, hurt us. Science 328:852–856
14. Ramakrishnan L (2012) Revisiting the role of the granuloma in tuberculosis. Nat Rev Immunol 12:352–366
15. Kim MJ, Wainwright HC, Locketz M, Bekker LG, Walther GB, Dittrich C, Visser A, Wang W, Hsu FF, Wiehart U, Tsenova L, Kaplan G, Russell DG (2010) Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. EMBO Mol Med 2:258–274
16. Lee W, VanderVen BC, Fahey RJ, Russell DG (2013) Intracellular Mycobacterium tuberculosis exploits host-derived fatty acids to limit metabolic stress. J Biol Chem 288:6788–6800
17. Eum SY, Kong JH, Hong MS, Lee YJ, Kim JH, Hwang SH, Cho SN, Via LE, Barry CE 3rd (2010) Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. Chest 137:122–128
18. Migliori GB, Hopewell PC, Blasi F, Spanevello A, Raviglione MC (2006) Improving the TB case management: the international standards for tuberculosis care. Eur Respir J 28:687–690
19. Nuermberger EL, Yoshimatsu T, Tyagi S, Williams K, Rosenthal I, O'Brien RJ, Vernon AA, Chaisson RE, Bishai WR, Grosset JH (2004) Moxifloxacin-containing regimens of reduced duration produce a stable cure in murine tuberculosis. Am J Respir Crit Care Med 170: 1131–1134
20. Shargie EB, Lindtjorn B (2005) DOTS improves treatment outcomes and service coverage for tuberculosis in South Ethiopia: a retrospective trend analysis. BMC Public Health 5:62
21. Gordon S, Rabinowitz S (1989) Macrophages as targets for drug delivery. Adv Drug Deliv Rev 4:27–47
22. Sacks LV, Pendle S, Orlovic D, Andre M, Popara M, Moore G, Thonell L, Hurwitz S (2001) Adjunctive salvage therapy with inhaled aminoglycosides for patients with persistent smear-positive pulmonary tuberculosis. Clin Infect Dis 32:44–49
23. Roy CJ, Sivasubramani SK, Dutta NK, Mehra S, Golden NA, Killeen S, Talton JD, Hammoud BE, Didier PJ, Kaushal D (2012) Aerosolized gentamicin reduces the burden of tuberculosis in a murine model. Antimicrob Agents Chemother 56:883–886
24. Hirota K, Hasegawa T, Hinata H, Ito F, Inagawa H, Kochi C, Soma G, Makino K, Terada H (2007) Optimum conditions for efficient phagocytosis of rifampicin-loaded PLGA microspheres by alveolar macrophages. J Control Release 119:69–76
25. Weibel ER (1963) Morphometry of the human lung. Springer, Berlin
26. Hickey AJ (2002) Delivery of drugs by the pulmonary route. In: Banker GS, Rhodes CT (eds) Modern pharmaceutics. Marcel Dekker, New York
27. Gonda I (1981) A semi-empirical model of aerosol deposition in the human respiratory tract for mouth inhalation. J Pharm Pharmacol 33:692–696
28. Byron PR (1986) Some future perspectives for unit dose inhalation aerosols. Drug Dev Ind Pharm 12:993–1015
29. Batycky RP, Hanes J, Langer R, Edwards DA (1997) A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. J Pharm Sci 86: 1464–1477
30. O'Hara P, Hickey AJ (2000) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization. Pharm Res 17:955–961
31. Makino K, Nakajima T, Shikamura M, Ito F, Ando S, Kochi C, Inagawa H, Soma G, Terada H (2004) Efficient intracellular delivery of rifampicin to alveolar macrophages using rifampicin-loaded PLGA microspheres: effects of molecular weight and composition of PLGA on release of rifampicin. Colloids Surf B Biointerfaces 36:35–42

32. Sharma R, Saxena D, Dwivedi AK, Misra A (2001) Inhalable microparticles containing drug combinations to target alveolar macrophages for treatment of pulmonary tuberculosis. Pharm Res 18:1405–1410
33. Ito F, Makino K (2004) Preparation and properties of monodispersed rifampicin-loaded poly(lactide-co-glycolide) microspheres. Colloids Surf B Biointerfaces 39:17–21
34. Atkins PJ (2005) Dry powder inhalers: an overview. Respir Care 50:1304–1312
35. Owens DR, Zinman B, Bolli G (2003) Alternative routes of insulin delivery. Diabet Med 20:886–898
36. Suarez S, O'Hara P, Kazantseva M, Newcomer CE, Hopfer R, McMurray DN, Hickey AJ (2001) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: screening in an infectious disease model. Pharm Res 18:1315–1319
37. Suarez S, O'Hara P, Kazantseva M, Newcomer CE, Hopfer R, McMurray DN, Hickey AJ (2001) Airways delivery of rifampicin microparticles for the treatment of tuberculosis. J Antimicrob Chemother 48:431–434
38. Tomoda K, Kojima S, Kajimoto M, Watanabe D, Nakajima T, Makino K (2005) Effects of pulmonary surfactant system on rifampicin release from rifampicin-loaded PLGA microspheres. Colloids Surf B Biointerfaces 45:1–6
39. Muttil P, Kaur J, Kumar K, Yadav AB, Sharma R, Misra A (2007) Inhalable microparticles containing large payload of anti-tuberculosis drugs. Eur J Pharm Sci 32:140–150
40. Sung JC, Garcia-Contreras L, Verberkmoes JL, Peloquin CA, Elbert KJ, Hickey AJ, Edwards DA (2009) Dry powder nitroimidazopyran antibiotic PA-824 aerosol for inhalation. Antimicrob Agents Chemother 53:1338–1343
41. Sharma R, Muttil P, Yadav AB, Rath SK, Bajpai VK, Mani U, Misra A (2007) Uptake of inhalable microparticles affects defence responses of macrophages infected with *Mycobacterium tuberculosis* H37Ra. J Antimicrob Chemother 59:499–506
42. Fiegel J, Garcia-Contreras L, Thomas M, VerBerkmoes J, Elbert K, Hickey A, Edwards D (2008) Preparation and in vivo evaluation of a dry powder for inhalation of capreomycin. Pharm Res 25:805–811
43. Dharmadhikari AS, Kabadi M, Gerety B, Hickey AJ, Fourie PB, Nardell E (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: a new approach to therapy of drug-resistant tuberculosis. Antimicrob Agents Chemother 57:2613–2619
44. Wong YL, Sampson S, Germishuizen WA, Goonesekera S, Caponetti G, Sadoff J, Bloom BR, Edwards D (2007) Drying a tuberculosis vaccine without freezing. Proc Natl Acad Sci U S A 104:2591–2595
45. Rogers TL, Johnston KP, Williams RO (2001) Solution-based particle formation of pharmaceutical powders by supercritical or compressed fluid CO_2 and cryogenic spray-freezing technologies. Drug Dev Ind Pharm 27:1003–1015
46. Yu ZS, Rogers TL, Hu JH, Johnston KP, Williams RO (2002) Preparation and characterization of microparticles containing peptide produced by a novel process: spray freezing into liquid. Eur J Pharm Biopharm 54:221–228
47. Meers P, Neville M, Malinin V, Scotto AW, Sardaryan G, Kurumunda R, Mackinson C, James G, Fisher S, Perkins WR (2008) Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic *Pseudomonas aeruginosa* lung infections. J Antimicrob Chemother 61:859–868
48. Vyas SP, Kannan ME, Jain S, Mishra V, Singh P (2004) Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. Int J Pharm 269:37–49
49. Fujiwara T, Konishi M, Chida S, Okuyama K, Ogawa Y, Takeuchi Y, Nishida H, Kito H, Fujimura M, Nakamura H, Hashimoto T, Surfactant-TA Study Group (1990) Surfactant replacement therapy with a single postventilatory dose of a reconstituted bovine surfactant in preterm neonates with respiratory distress syndrome: final analysis of a multicenter, double-blind, randomized trial and comparison with similar trials. Pediatrics 86:753–764
50. Pandey R, Khuller GK (2005) Antitubercular inhaled therapy: opportunities, progress and challenges. J Antimicrob Chemother 55:430–435
51. Aderem A, Underhill DM (1999) Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 17:593–623

52. Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422:37–44
53. Hansen CG, Nichols BJ (2009) Molecular mechanisms of clathrin-independent endocytosis. J Cell Sci 122:1713–1721
54. Sahay G, Alakhova DY, Kabanov AV (2010) Endocytosis of nanomedicines. J Control Release 145:182–195
55. Swanson JA, Watts C (1995) Macropinocytosis. Trends Cell Biol 5:424–428
56. Tamaru M, Akita H, Fujiwara T, Kajimoto K, Harashima H (2010) Leptin-derived peptide, a targeting ligand for mouse brain-derived endothelial cells via macropinocytosis. Biochem Biophys Res Commun 394:587–592
57. Bhattacharya S, Roxbury D, Gong X, Mukhopadhyay D, Jagota A (2012) DNA conjugated SWCNTs enter endothelial cells via Rac1 mediated macropinocytosis. Nano Lett 12:1826–1830
58. Mukhopadhyay S, Herre J, Brown GD, Gordon S (2004) The potential for toll-like receptors to collaborate with other innate immune receptors. Immunology 112:521–530
59. Prescribing information. Janssen Products, LP, http://www.doxil.com/shared/product/doxil/prescribing-information.pdf. Accessed 6 Sept 2013
60. Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, Corstens FH, Boerman OC (2000) Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J Pharmacol Exp Ther 292:1071–1079
61. Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, Kiwada H (2006) Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. J Control Release 112:15–25
62. Ishida T, Masuda K, Ichikawa T, Ichihara M, Irimura K, Kiwada H (2003) Accelerated clearance of a second injection of PEGylated liposomes in mice. Int J Pharm 255:167–174
63. Hirota K, Hasegawa T, Nakajima T, Inagawa H, Kohchi C, Soma G, Makino K, Terada H (2010) Delivery of rifampicin-PLGA microspheres into alveolar macrophages is promising for treatment of tuberculosis. J Control Release 142:339–346
64. Hirota K, Terada H (2012) Endocytosis of particle formulations by macrophages and its application to clinical treatment. In: Ceresa B (ed) Molecular regulation of endocytosis. InTech, Rijeka
65. Phalen RF (1976) Inhalation exposure of animals. Environ Health Perspect 16:17–24
66. Dorato MA (1990) Overview of inhalation toxicology. Environ Health Perspect 85:163–170
67. Warheit DB, Carakostas MC, Hartsky MA, Hansen JF (1991) Development of a short-term inhalation bioassay to assess pulmonary toxicity of inhaled particles: comparisons of pulmonary responses to carbonyl iron and silica. Toxicol Appl Pharmacol 107:350–368
68. Costa DL, Lehmann JR, Harold WM, Drew RT (1986) Transoral tracheal intubation of rodents using a fiberoptic laryngoscope. Lab Anim Sci 36:256–261
69. Remie R, Bertens APMG, van Dongen JJ, Rensema JW, van Wunnik GHJ (1990) Anaesthesia of the laboratory rat. In: van Dongen JJ, Remie R, Rensema JW, van Wunnik GHJ (eds) Manual of microsurgery on the laboratory rat, part I. Elsevier Science Publishers, Amsterdam
70. Bivas-Benita M, Zwier R, Junginger HE, Borchard G (2005) Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. Eur J Pharm Biopharm 61:214–218
71. Tronde A, Baran G, Eirefelt S, Lennernäs H, Bengtsson UH (2002) Miniaturized nebulization catheters: a new approach for delivery of defined aerosol doses to the rat lung. J Aerosol Med 15:283–296
72. Cannon WC, Blanton EF, McDonald KE (1983) The flow-past chamber: an improved nose-only exposure system for rodents. Am Ind Hyg Assoc J 44:923–928
73. Kaur J, Muttil P, Verma RK, Kumar K, Yadav AB, Sharma R, Misra A (2008) A hand-held apparatus for "nose-only" exposure of mice to inhalable microparticles as a dry powder inhalation targeting lung and airway macrophages. Eur J Pharm Sci 34:56–65
74. Wong BA (2007) Inhalation exposure systems: design, methods and operation. Toxicol Pathol 35:3–14
75. Garcia-Contreras L, Sung JC, Muttil P, Padilla D, Telko M, Verberkmoes JL, Elbert KJ, Hickey AJ, Edwards DA (2010) Dry powder PA-824 aerosols for treatment of tuberculosis in guinea pigs. Antimicrob Agents Chemother 54:1436–1442

76. Verma RK, Kaur J, Kumar K, Yadav AB, Misra A (2008) Intracellular time course, pharmacokinetics, and biodistribution of isoniazid and rifabutin following pulmonary delivery of inhalable microparticles to mice. Antimicrob Agents Chemother 52:3195–3201
77. Lombry C, Bosquillon C, Preat V, Vanbever R (2002) Confocal imaging of rat lungs following intratracheal delivery of dry powders or solutions of fluorescent probes. J Control Release 83:331–341
78. Kawamoto T (2003) Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. Arch Histol Cytol 66:123–143
79. Hirota K, Kawamoto T, Nakajima T, Makino K, Terada H (2013) Distribution and deposition of respirable PLGA microspheres in lung alveoli. Colloids Surf B Biointerfaces 105:92–97
80. Dolovich M, Labiris R (2004) Imaging drug delivery and drug responses in the lung. Proc Am Thorac Soc 1:329–337
81. Pitcairn GR, Newman SP (1998) Radiolabellling of dry powder formulations. In: Dalby RN, Byron PR, Farr SJ (eds) Respiratory drug delivery VI. Interpharm Press, Buffalo Grove
82. Garcia-Contreras L, Sethuraman V, Kazantseva M, Godfrey V, Hickey AJ (2006) Evaluation of dosing regimen of respirable rifampicin biodegradable microspheres in the treatment of tuberculosis in the guinea pig. J Antimicrob Chemother 58:980–986
83. Yoshida A, Matumoto M, Hshizume H, Oba Y, Tomishige T, Inagawa H, Kohchi C, Hino M, Ito F, Tomoda K, Nakajima T, Makino K, Terada H, Hori H, Soma G (2006) Selective delivery of rifampicin incorporated into poly(DL-lactic-co-glycolic) acid microspheres after phagocytotic uptake by alveolar macrophages, and the killing effect against intracellular *Mycobacterium bovis* calmette-guerin. Microbes Infect 8:2484–2491
84. Garcia-Contreras L, Fiegel J, Telko MJ, Elbert K, Hawi A, Thomas M, VerBerkmoes J, Germishuizen WA, Fourie PB, Hickey AJ, Edwards D (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. Antimicrob Agents Chemother 51:2830–2836
85. Verma RK, Germishuizen WA, Motheo MP, Agrawal AK, Singh AK, Mohan M, Gupta P, Gupta UD, Cholo M, Anderson R, Fourie PB, Misra A (2013) Inhaled microparticles containing clofazimine are efficacious in treatment of experimental tuberculosis in mice. Antimicrob Agents Chemother 57:1050–1052
86. Helke KL, Mankowski JL, Manabe YC (2006) Animal models of cavitation in pulmonary tuberculosis. Tuberculosis (Edinb) 86:337–348

Chapter 6
Hepatic Targeting: Physiological Basis and Design Strategy

Anisha A. D'Souza, Vishvesh M. Joshi, and Padma V. Devarajan

6.1 Introduction

The mammalian liver plays a stalwart role in the metabolism of carbohydrates, fats, and proteins and detoxification of organic by-products, cellular debris, drugs, pesticides, xenobiotics, foreign particles, etc. from the systemic circulation. It is also involved in anabolism of cholesterol, steroid hormones, biochemicals, and proteins. Being the largest and strategically located internal organ with a plethora of functions, it is prone to many contaminants, injuries, and disorders. Diseases afflicting the liver continue to be the fifth most common cause of death and are ever-increasing [1]. Grave hepatic disorders range from liver fibrosis or cirrhosis, fulminant hepatitis or viral hepatitis (A, B, C, D, E, G), primary liver cancer, hepatic cholangiocarcinoma, severe congenital liver failures, metabolic genetic disorders, and hepatocellular carcinoma (HCC). HCC has one of the lowest (1-year) survival rates among all cancers [2] with about 5,00,000 new cases diagnosed every year, especially in developed nations [3]. While surgical interventions are resorted to in benign cancers, chemotherapy is the preferable treatment in cancers [4].

Most drugs achieve high hepatic concentration after administration. However, drugs for treating liver disorders have often experienced circumscribed success with a high relapse rate, due to limited efficacy and poor sensitivity at conventional doses. Dose escalation is often hindered by patient tolerability, hepatic and off-target safety concerns, and high resistance due to efflux pumps (P-glycoprotein) which limit their efficacy [5]. Moreover, different hepatic conditions obligate high degree of specificity and accumulation within the proper intrahepatic cells for addressing optimal therapeutic potential. At the receptor level, the treatment varies with varying

A.A. D'Souza • V.M. Joshi • P.V. Devarajan (✉)
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (Elite status), Deemed University, Matunga, Mumbai, India
e-mail: pvdevarajan@gmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_6

harboring sites of disorders. This has fueled an exigent need for effective, safe, yet affordable liver targeted drug delivery systems.

The past two decades have seen an increase in nanotechnology based liver targeted drug delivery, which relies on altered biodistribution for enhanced therapeutic efficacy. The unprecedented development relies on the unique size and surface characteristics, overcoming anatomical and physiological barriers coupled with enhanced penetrability. Enabling early diagnosis is yet another feature [6]. Nanoparticulates in the size range of 50–250 nm are easily accumulated in the liver [7]. Besides, engineering of these nanoparticulates for site specific hepatic delivery is amenable.

The present chapter comprehensively reviews the liver architecture, various cell types, and approaches for targeted drug delivery to liver. Active and passive targeting strategies with a focus on the hepatic receptors are detailed.

6.2 Liver Architecture: Normal and Pathological State

The liver is a histologically complex organ with four types of substantial target cells—hepatocytes, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells. The histological unit of the liver is the lobule. Primarily, about 80 % of the liver comprises parenchymal cells (PC) or hepatocytes (Fig. 6.1) [8]. The hepatocytes and discontinuous hepatic sinusoidal endothelial cells (SEC) are physically demarcated by the space of Disse also known as peri-sinusoidal space containing dispersed fat containing lipocyte, or hepatic stellate cells (HSC). Plasma is preferentially filtered through the sinusoids into the space of Disse, and provides body lymph.

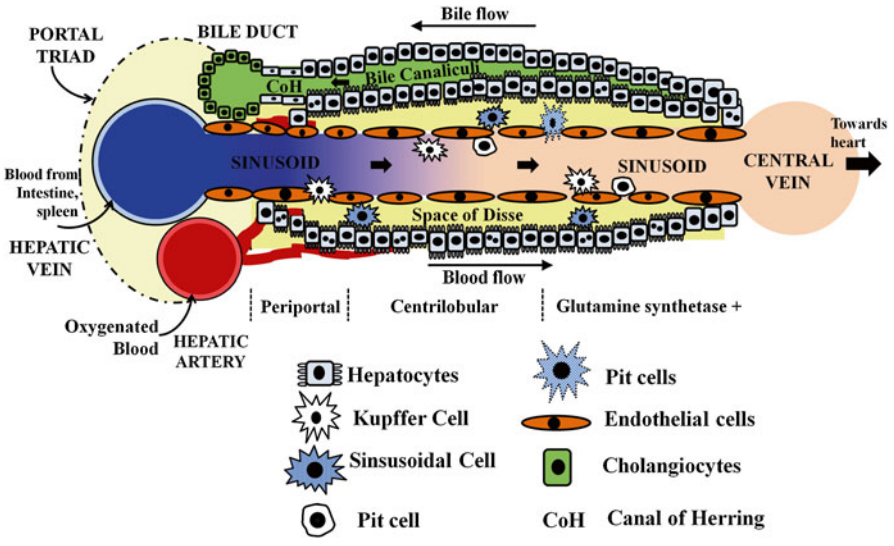

Fig. 6.1 Pictorial representation of a liver lobule

Hence, exchange of nutrients, proteins, and wastes between hepatocytes and blood occurs in this microenvironment of space of Disse. In obliteration of this space as in alcoholic liver diseases, uptake by hepatocytes is hindered. The widened space of Disse increases resistance to sinusoidal blood flow thereby raising portal pressure [9]. The Kupffer cells (KC) or the resident non-parenchymal liver macrophages (~18 %) are located along the luminal side of the EC in the sinusoidal area with no specialized contacts [10]. Also located on the endothelial lining are the pit cells that correspond to large granular lymphocytes with natural killer activity. The RES (reticuloendothelial system) of liver consists of SEC and KC [11, 12]. The liver receives oxygen rich blood through the hepatic artery and hepatic portal vein (shunted capillaries from spleen and intestine). The space connecting the biliary ductules and hepatocytes is the Canal of Hering [13]. Canal of Hering plays an important role in carcinogenesis [14].

6.2.1 Reticuloendothelial System (RES) Cells of the Liver

Reticuloendothelial system (RES) is a part of human body defense, derived from bone marrow contributing to both nonspecific and specific immunity. Till recently the RES was considered synonymous with the mononuclear phagocytic system (MPS). However, it has now been established that RES constitute both wandering and sessile phagocytic cells, e.g., monocytes, SEC, KC, polymorphonuclear leucocytes, dendritic cells, histiocytes. whereas the MPS is restricted only to macrophages like KC. The role of the liver RES can be summarized as follows:

- Engulfment and ingestion (phagocytosis) of abnormal cells, pathogens and foreign substances.
- Presentation of antigens or foreign invaders to lymphocytes which secrete antibodies.

6.2.1.1 Kupffer Cells (KC)

KC form only 6.5 % of liver volumes, but contribute to 80–90 % of tissue macrophages present in the human body [15]. KC are found in high number in rats over ~20 months old [16] following partial hepatectomy, or a single intravenous injection of zymosan [17] and in alcohol related hepatitis and liver diseases [18]. Depletion of KC is seen on administration of gadolinium chloride [19], clodronate liposomes [20], and in HCC [21].

Phagocytosis of IgG-coated erythrocytes also decreases the complement receptor of KC [22]. KC exhibit abundant lysosomes and pronounced phagocytosis as they are specialized macrophages of the reticuloendothelial system (RES). The typical macrophage activity of KC plays a crucial role in innate immune defense, ischemia, resection, acute and chronic responses to toxic compounds and removal of particulate,

damaged debris, bacterial and viral infections, endotoxins, etc. [23]. Activation of KC produces pro-inflammatory mediators, e.g., nitric oxide, prostanoides, signaling molecules (cytokines, TNF-alpha), macrophage colony-stimulating factor, reactive oxygen species, other growth factors (innate immune defense) and prevents liver inflammation [15]. Balance of these secretions is necessary to maintain a harmonious environment for the hepatic cells and the extracellular matrix. Exposure to lipopolysaccharide endotoxins leads to damage of hepatocytes and liver injury [24]. KC is responsible for pathogenesis of non-alcoholic steatohepatitis, viral hepatitis, fibrosis, intrahepatic cholestasis, alcoholic liver disease, rejection of liver during liver transplantation, etc. [18]. Besides macrophagic activity, KC plays a key role in arresting circulating tumor cells and controlling metastasis [25] as well as in the clearance of erythrocytes by scavenger receptors [26].

6.2.1.2 Sinusoidal Endothelial cells (SEC)

The roles of KC and SEC exhibit some overlap and are at times controversial [11, 12]. SEC are considered dormant of phagocytosis; however, on impairment of KC, SEC acquire phagocytotic competence. SEC constitutes about 40 % of hepatic cells, and represents a barrier between blood and hepatocytes. SEC form small fenestrations (50–200 nm)and are grouped together to form sieve plates permitting filtration, thereby allowing diffusion of many substances but not of chylomicron size (80–500 nm) [27]. Compared to KC, SEC uptake colloids of size <0.23 μm or soluble materials, while KC can take up larger particles up to 15 μm [28]. However, impairment of KC facilitates uptake of large particles by SEC [11, 12]. Contrary to the above, colloidal carbon is reported to preferentially accumulate in KC upon intravenous injection. Smaller colloid particles fail to reach SEC due to aggregation in plasma or adherence to platelets resulting in their phagocytosis by KC [29]. In non-alcoholic fatty liver disease, simple infiltration of fat and chylomicrons is enhanced and accumulated in liver. Liver SEC exhibit huge receptor endocytotic capacity for extracellular matrix components, e.g., hyaluronic acid, collagen (especially in SEC not expressing Endo180) and play a major role in metabolism of the extracellular matrix [30, 31]. Damage to SEC is associated with graft versus host disease, veno-occlusive disease and sepsis [32]. Deposition of extracellular matrix leads to thickening of SEC causing defenestration of SEC followed by fibrosis. Overall, SEC plays an important role in regulation of hemostasis, inflammatory reactions, microcirculation, and immunity [33, 294].

6.2.1.3 Hepatic Stellate Cells (HSC) (Ito Cells or Lipocytes)

HSC house 80 % of retinoid found in the entire body. Cellular retinol-binding protein, type 1 (CRBP) binds to retinol and undergoes receptor mediated endocytosis of the complex complex containing retinol and Retinol Binging Protein (RBP), to maintain plasma retinol [34]. Besides these, platelet-derived growth factors, epidermal and fibroblast activation protein, adhesion molecules, cytokines, vascular cell integrins, etc. activate HSC [35].

Under normal physiological conditions, HSC are in the quiescent state [36]. In the activated state they act as antigen presenting cell and stimulate proliferation of natural killer T cells [37]. HSC secrete fibronectin and vascular endothelial growth factor stimulating production of nitric oxide. As a result retinol is lost from the cell and HSC undergo morphological change. This leads to increased proliferation and trans-differentiation to fibrogenic myofibroblast-like cells [38] which secrete collagen scar tissue and fibrogenic and inflammatory cytokines (extracellular matrix). Fibrosis and cirrhosis therefore result [39].

6.2.1.4 Pit Cells/Large Granulated Lymphocytes

Pit cells, large granulated lymphocytes are present in lower numbers, approximately 10 % of KC. They function as natural killer cells. These cells are 0.2–0.5 μm in diameter and majorly contain acid phosphatase. Pit cells possess much higher cytotoxic activity and higher grade of activation with diverse immune phenotypic features. Situated in the sinusoidal lumen, their cytoplasmic processes adhere to KC and with microvilli of hepatocytes through the endothelial sieve. When triggered by biological response modifiers, proliferation of pit cells occurs with migration towards the Space of Disse to exhibit viricidal activity. Interleukin-2 released during viral infections and neoplasms is also known to trigger such transit [40]. Pit cells exhibit spontaneous antitumor activity by adhering to tumor cells [41] and also kill hepatitis virus-infected cells [42].

6.2.2 Non-reticuloendothelial System Cells (RES) of Liver

6.2.2.1 Hepatocytes

Hepatocytes are principally involved in the metabolism of carbohydrates, fat, and proteins as well as in secretion of bile, clotting factors, and cholesterol and protein transporters. They comprise ~80 % of liver volume with distinct nucleoli, both rough and smooth endoplasmic reticulum, mitochondria, and Golgi apparatus [43]. These highly metabolic active cells break down toxic chemicals, drugs and hormones which are easily eliminated from circulation. This is also known as “first pass effect.” Hepatocytes lining the bile canaliculi possess numerous Golgi vesicles. Hepatocytes are critical in synthesis of molecules supporting homeostasis of glucose and cholesterol and maintaining energy levels. They are storage sites for glucose, vitamins (A, D, E, K, folate, B12), and minerals (Cu, Fe). Metabolic activities in the liver lobule although compartmentalized are highly integrated. The periportal hepatocytes involve themselves in gluconeogenesis and glycogenolysis, while the centrolobular hepatocytes are responsible for glycogen synthesis and glycolysis. The glutamine synthetase positive centrolobular hepatocytes are involved in metabolism of ammonia and the periportal hepatocytes are responsible for removal of ammonia. The microenvironmental signal for the differential positions is the differences in

oxygen gradient [44]. Hepatocytes are well differentiated with high and unlimited capacity of replication and longevity. The rapid growth of liver (60–70 %) after resection is mainly dependent on hepatocyte proliferation and hyperplasia [45]. Extensive proliferation of hepatocytes and cellular damage is observed in liver injury, hepatocellular carcinoma, chronic hepatitis, and exposure to certain chemicals [46] and continues till cirrhosis. Decreased hepatocyte number is seen in chronic consumption of ethanol [47], decreased hepatocyte growth factor activity and impaired liver regeneration, ischemia–reperfusion, etc. [48].

6.2.2.2 Biliary Cells: Cholangiocytes

The biliary cells are a part of hepatic cell lineage developed during embryogenesis along with hepatoblasts and form 1 % of the liver [49]. They exhibit heterogeneity in both morphological functions, extending from the liver hilum to the bile duct. The function and phenotype properties vary with hepatocytes though derived from the same lineage. The biliary epithelial cells maintain contact with the hepatoblasts and express markers for hepatocytes (albumin and alpha-fetoprotein) and bile duct epithelium (cytokeratins 7 and 19, carcinoembryonic antigen, carboanhydrase, glutamyl transpeptidase) [14, 40]. They regulate bile formation, liver inflammatory process, fibrogenesis, and angiogenesis. Bile duct cells are affected in bile duct cancer (cholangiocarcinoma) predominantly observed in women [50].

6.2.2.3 Stem Cells

Recently, an unsettled discussion has been the detection of progenitor cells/ hepatic stems. The origin of these cells at the junction of the hepatic cords (Canal of Hering) and bile ducts has been debated as either migration from bone marrow to liver or being the real hepatic resident cells [51]. Stem cells are non-specialized cells with the abilities of self-renewable, limitless proliferation and resistance to chemotherapy. These stem cells generate oval cells on exposure to carcinogens with dual characteristic of hepatocyte and biliary cells, bipotential progenitor cells, which can generate hepatocytes and bile duct cells when the hepatocytes and cholangiocytes fail to regenerate [14]. Mutations in the stem cells are suggested to be responsible for growth and maintenance of cancer [52–54]. Research in hepatic stem cells is in its infancy with size and morphology yet not clear.

6.3 Hepatic Targeting

Targeting to the liver can be achieved through direct intraportal, intra-tumoral, intra-arterial route injection. Direct administration to the site prevents unnecessary exposure to other non-target organs. Retrograde intrabiliary infusion for genetic

delivery of nanoparticulates or complexes has been one way wherein the hepatocytes could be specifically targeted easily via the biliary system [55–57]. The entire process necessitates the need for cannulation [58]. Targeting to the liver could be achieved by generalized organ based targeting or could be directed to one or more cell types detailed above. Approaches to target to the liver would essentially be dictated by the cells being targeted and could be achieved using two strategies, active or passive targeting. The practical approaches for passive and active targeting to the liver are detailed below.

6.3.1 Passive Targeting

Passive targeting relies on the basic defense mechanism of the RES to target foreign invaders like bacteria, viruses, etc., and this strategy can be widely explored for conditions wherein the RES is the target site of action [59, 60]. Understanding the conditions that trigger such targeting provides useful information to design passive targeting strategies outside the RES. The processes responsible for RES uptake are opsonization and phagocytosis.

6.3.1.1 Opsonization and Phagocytosis

Opsonization is fouling of invading particulates by deposition of plasma proteins mainly fibrinogens, fibronectin, lipoproteins, etc. [61]. Once opsonized, the foreign object or nanoparticulates are activated, recognized, and engulfed by macrophages via phagocytosis [61]. Opsonization of particulates by complements (C3, C4, and C5) and immunoglobulins makes the particulates more recognizable by the KC. Phagocytosis is initiated by attachment of the foreign body with the KC, followed by invagination and spreading of cell membrane covering the particle to form a vacuole called phagosome. Phagosomes coalesce with intracellular organelles to mature into phagolysosomes. Phagolysosomes have an acidic environment with many digestive proteins which finally degrades the internalized material. Phagocytosized material is eliminated by exocytosis. In case the particulate cannot be digested, it remains sequestered in residual bodies within the cell. The process of phagocytosis can be explained as given in Fig. 6.2.

Phagocytosis is a nonspecific uptake mechanism influenced by many factors such as shape, size, charge, rigidity, etc. [62].

1. Particle size
   Size and radius of particles affect the biodistribution profile and internalization by KC. Optimal phagocytosis occurs with particles of 1 and 3 μm. Smaller particles (<35 nm diameter) escape the interaction contacts with KC but are easily removed by the kidney and provide more easy access to hepatocytes [63]. Nanocarriers with a particle size limit of 80 nm have physical access to hepatocytes [64]. Targeting to hepatocytes necessitates design particles of less than 100 nm

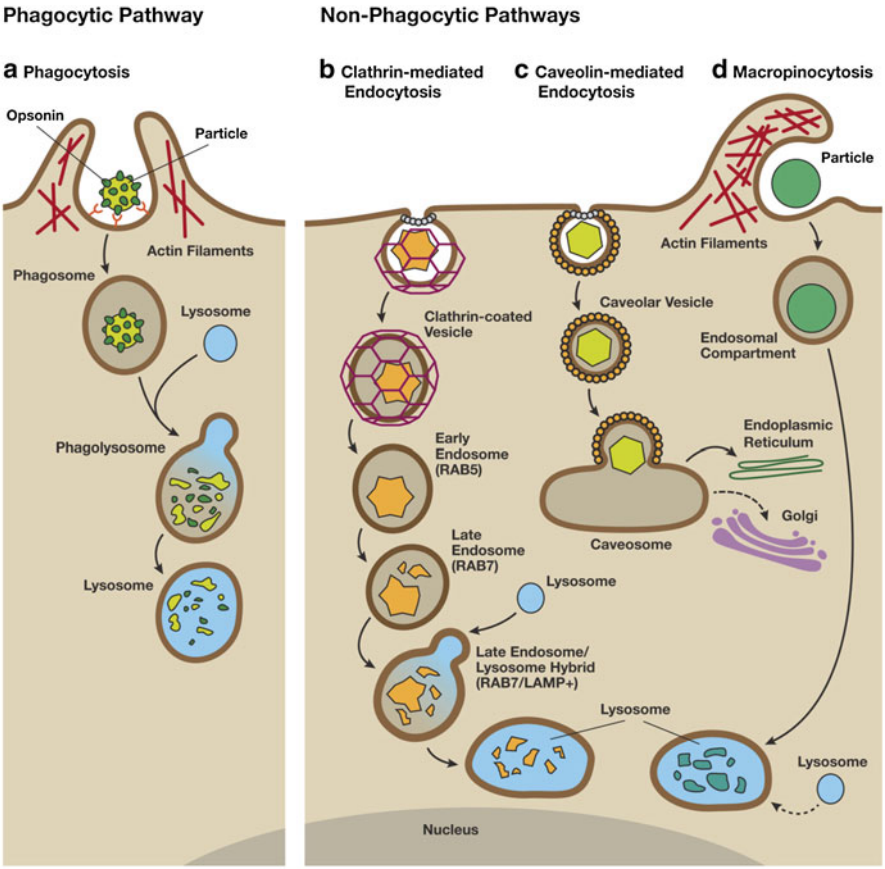

Fig. 6.2 Different uptake mechanisms for particles (reproduced from [279])

to diminish KC uptake. Large sized rigid particulates up to a size of 20 μm or those with three times the volume of macrophages are removed by the RES system, typically liver and spleen macrophages [65, 66]. Excessively larger particles cannot be internalized easily as it requires strong and extensive cytoskeleton remodeling [67]. The upper size limit for phagocytosis has been determined around 20 μm in vitro or whenever the size exceeds more than three times than that of KC [68]. Liu et al. [69] investigated the biodistribution of different sized (30–400 nm) liposomes. Particles greater than 250 nm in size irrespective of PEGylation are rapidly removed from the RES [70]. Excessive and maximum stretching of KC membrane causes frustrated phagocytosis wherein the system is not fully engulfed [71].

2. Surface charge
   Cationic nanoparticulates with a zeta potential >25 mV amplify complement activation and deposition of opsonins than those below 15 mV [72, 73].

Self-aggregation and opsonization of nanoparticulates with anionic serum protein causes passive accumulation in the RES cells. Neutral charged nanocarriers decrease the KC uptake [74]. Positively charged nanoparticles therefore exhibit a higher cell uptake than hydrophilic neutral or negatively charged particles [75]. Long half-life of anionic carriers could be due to less opsonin adsorption [76]. Intravenous administration of extracellular superoxide dismutase plasmid as polycationic liposome resulted in reduced peroxidation of lipids and enhanced levels of hepatic glutathione and serum superoxide dismutase [77]. Coatings of hyaluronic acid prolonged circulation times [78]. Nucleic acids have been successfully delivered to hepatocytes through cationic and PEGylated liposomes (80–100 nm) with higher suppression of HBV, attributed to longer half-life of nucleic acids [79].

3. Particle shape
   The effect of shape on phagocytosis has been recognized and particle shape has been recently reported as an influencing factor for MPS uptake [80, 81]. The ability of irregular shaped polymer–lipid hybrid nanoparticles (LIPOMER) to bypass the KC and accumulate in the spleen has been demonstrated (~400 nm) [82]. Non-spherical shaped particles bypassed phagocytosis due to incomplete actin structure formation. Uptake of rod shaped particles is unachievable if they macrophages attack them on their major axis [81]. Likewise oblate (disk-like) particles effectively adhered to cell surfaces compared to spherical particles of comparable volume to bypass phagocytosis [83]. High aspect ratios (i.e., ratio of larger surface dimension over smaller surface dimension) hinder actin membrane spreading and hence internalization [67]. Spherical nanoparticulates of sub 100 nm displayed higher uptake than rod shaped particles [84].
4. Flexibility and deformability
   The effect of flexibility and deformability on uptake by macrophages is also cited [85]. Stiffness of the particles influences the shape of phagocytic cup formed after activation of actin recruitment [67]. Particles should be either small or deformable to be able to penetrate through sinusoidal fenestrations for hepatocyte targeting. Reports of deformable nanoparticulates sized 400 nm being extravasated via forced extrusion mechanism bypassing KC and RES cells enabled localization in the hepatic parenchyma [86]. Fc-receptor mediated phagocytosis internalizes large rigid opsonized particles preferentially over softer particles. It influences the activation of actin recruitment to shape the phagocytic cup [87].
5. Hydrophilicity
   Particles with a hydrophobic surface are rapidly removed from circulation. PEGylation masks the particle appearing more like water body and prevent RES uptake [74]. Surface modification with hydrophilic coatings enables particles to masquerade as water bodies and by pass the RES. The hepatic B virus is considered to be a best example of stealth, as it escapes the RES [88]. PEGylated tamoxifen nanoparticles bypassed the liver compared to non-PEGylated nanoparticles [89]. Hydrophilic coating recommended to enable decreased KC uptake with higher parenchymal uptake include dextran, phosphatidylinositol,

monosialoganglioside, pullulan, poloxamers, polyvinylpyrrolidone, and cellulose derivatives [90–92]. This is the popularly known "Stealth" technology. Stealth technology can be exploited for effective delivery of drugs to the liver in infections and inflammations. Under such stress, increase in vascular permeability or increase in dimension of fenestrations enable leukocyte extravasation and accumulation at the inflamed site. Thus, particulates of lower dimensions can easily pass through these pores which are generally inaccessible.

6.3.2 Active Targeting

In active targeting, therapeutics are transported selectively and specifically to relevant cells with the help of ligands through receptor mediated endocytosis [93] or through stimuli responsive nanocarriers, e.g., temperature, ultrasound, magnetic field [94]. Ligands such as carrier proteins, metabolites, saccharides, peptides, vitamins, lectins, hormones, antibodies, aptamers, neurotransmitters, etc. are grafted on nanoparticulates and thus selectively target specific receptors. Addressing drug delivery systems can prevent non-desired accumulation in the body and exert precise effects especially on cells with low expression [95]. Designing strategies to target receptors thus holds intriguing promise in therapeutic interventions, bypassing multidrug resistance [96].

Delivery of charged molecules and genetic materials intracellularly is better facilitated by nanoparticulates attached with fusogenic agents or ligands for active targeting [97]. Besides, the higher the valency of binding, the higher the binding potential [98]. Readers are requested to make a note that the receptors dealt below are mostly transmembrane in nature rather than intracellular receptors as they would play an important role in transportation of carriers to intracellular environment of cell.

6.3.3 Receptor Mediated Active Targeting

Different receptors are present on cell membranes responsible for specific interaction with neighboring cells. These receptors also facilitate specific interaction with carrier system. Receptor mediated endocytosis follows adsorptive pinocytosis. Mechanisms of binding and internalization vary from clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis depending on the size of the endocytic vesicle, the nature of the cargo and the mechanisms of vesicle formation [99]. For details on these uptake mechanisms readers are directed to the following references [91, 100, 101]. Macropinocytosis is a transient process while micropinocytosis (clathrin-dependent, caveolae mediated, and clathrin- and caveolae-independent endocytosis) is a constitutive pathway. Clathrin-coated vesicles and macropinosomes fuse with endolysosomes whereas caveolae-coated vesicles can escape endolysosomes and lead to direct exocytosis [102].

Several endocytotic mechanisms often take place simultaneously [91]. Clathrin-mediated endocytosis is one of the best characterized and widely studied endocytosis pathways. The best known receptors adopting this mechanism are ASGP-R, low density lipoprotein receptor, epidermal growth factor receptor (tyrosine kinase receptor), β2-adrenergic receptors, etc.

Clathrin-mediated endocytosis a common pathway for the internalization of a variety of ligand–receptor complexes. These processes are relatively slower than that of phagocytosis. As for phagocytosis, binding of ligand to the receptor is also dependent on size, geometry of ligand, charge, density of ligands, etc. [103]. Various receptors found on different types of liver cells are summarized in Table 6.1.

Table 6.1 Receptors on hepatic cells and their ligands

Receptor	Ligand	References
Kupffer cells		
Mannose/*N*-acetylglucosamine receptors	Mannose and *N*-acetylglucosamine	[280]
Fucose recognition receptors	Fucose	[105, 149, 281]
Fc receptors (FcγII-B2)	Antibodies/IgG	[282, 283]
Scavenger receptors (SR-BI, MARCO, dSR-C1, CD36, 95 kDa receptor Macrosialin)	Modified or acetylated LDL, polyanionic, lipopolysaccharides	[113, 284, 285]
Cannabinoid receptors (CB2)	Endocannabinoid	[195]
LDL receptors	LDL	[179, 286]
Fibronectin receptors		[287]
Sinusoidal Endothelial cells		
Scavenger receptors (SR-AI, AII, B, H)	Oxidized LDL, polyinosinic and polyguanosinic acid, polyanionic ligands	[288, 289]
Mannose receptors/*N*-acetyl glucosamine receptors	Mannose, lysosomal enzymes, tissue plasminogen activator, immune complex	[11, 12, 31]
Fc receptor	Antibodies/IgG	[119, 290, 291]
Stabilin receptor	Hyaluronic acid, chondroitin sulfate	[289, 292]
Collagen receptor	Denatured collagens	[40]
Laminin receptor	Laminin/nidogen	[40]
Hepatic stellate cells		
Retinol-binding protein receptor	Retinol	[187]
Cytokine receptors	–	[35, 293]
Transferrin receptor	Transferring	[294]
Growth factors—platelet-derived, cell–matrix interactions, epidermal and fibroblast	C*SRNLIDC* peptide, Arg-Gly-Asp (RGD) peptide	[299]
Tyrosine kinase receptors	–	[295, 296]
Uroplasminogen receptors	–	[297, 298]
Vasopressin receptors		[294, 299]
Integrin/complement receptors (CR1, CR3, CR4 C3b and C1q)	Opsonized components	[22, 300, 301]

(continued)

Table 6.1 (continued)

Receptor	Ligand	References
Hepatocytes		
Asialoglycoprotein receptors	Galactose terminated glycoproteins, arabinogalactan, pullulan, sitoG	[146, 302]
Glycyrrhizin(GL)/glycyrrhetinic acid (GA) receptors	Glycyrrhizin, glycyrrhetinic acid	[303]
HDL receptors	High density lipoprotein	[304]
LDL receptors	Low density lipoprotein	[305–307]
Scavenger receptors (SR-BI, CD36)	Native and modified lipoproteins, anionic phospholipids, apoptotic cells	[308, 309]
Transferrin receptors	Transferrin and its derivatives	[310–312]
Insulin receptors	Insulin analogues	[313, 314]
Ionotrophic purinergic receptors (P2X)	–	[315]
Glucagon-like peptide-1 receptor (GLP-1)	Exendin-4	[202]
Cannabinoid receptors (CB1)	Endocannabinoids	[195, 316]

6.3.3.1 Kupffer Cells

(a) Mannose receptors or *N*-acetyl glucosamine receptor/GlucNAc R
Mannose/*N*-acetylglucosamine receptor recognizes and clears off glycoproteins with mannose, glucose, and N-acetylglucosamine residues in exposed positions regardless of SEC and Kupffer cell. Mannose receptors are major receptor responsible for removal of denatured collagen from blood [31]. Mannosylated – human serum albumin selectively targets the KC and the EC [104–106]. Anti-inflammatory actives such as dexamethasone, immunosuppressive, enzymes like superoxide dismutase in chronic or acute hepatic inflammatory disorders, alcohol-induced hepatitis have been actively targeted to KC through mannose receptors [106, 107]. A drawback of targeting mannose receptors is activation of signaling processes sensitizing the immune system [108, 109]. Genetic delivery through mannose receptors have also been reported [93]. Though mannose receptor possesses eight carbohydrate recognition domains, only one is actively involved in binding. Mannose receptors differ from ASGP-R receptors in terms of binding. High mannose glycans are poor ligands for the mannose receptor. Liposomes possessing mannosylated ligands have exhibited enhanced targeting to macrophages, both in vitro and in vivo, than the non-ligand ones [110]. Lei Dong mentions the presence of one more macrophage lectin β-glucan receptor which binds glucose or glucan polymers. Hence, chitosan, a glucosamine polymer has considerable affinity for macrophages [111].

(b) Fucose receptors
Fucose receptors are responsible for clearance of glycoproteins bearing terminal fucose sugar. In vitro studies revealed that fucose and mannose receptor both

regulate uptake of fucosylated BSA. Nevertheless, fucosylated BSA is more Kupffer cell-selective because it exhibited a lower sinusoidal endothelial cell uptake than mannosylated BSA [105]. Fucosylation is more commonly explored in diagnosis than in therapeutics [112].

(c) Scavenger receptors
Scavenger receptors on KC constitute the scavenger receptors class SR-A (type I, II, and MARCO) and SR-B (type I, CD36, and CD68/macrosialin) [113]. It is known to be downregulated in animal models of Nonalcoholic steatohepatitis [114]. The CD68 is partially expressed in endolysosomal compartments and also on also on the transmembrane of macrophages [115]. Plasma proteins are removed from circulation by inducing a negative charge on its surface by succinylation to the lysine groups [26, 116]. The coated pits create a cationic surface charge permitting endocytosis of highly negatively charged molecules [117]. Particles up to 0.23 μm can be easily internalized [28]. Among the various receptors, scavenger receptors class A binds to varied polyanionic ligands but with varied affinity [113]. Expression of SR-A varies with the presence of ligands; lipopolysaccharides decrease the expression while oxidized LDL increases the expression [118].

Ligands such as fucoidan, polyinosinic acid, phosphatidylserine, oxidized low-density lipoprotein have a high affinity for scavenger receptors. Scavenger receptors play a major role in discrimination between foreign and self [118]. Weak negatively charged compounds show only a small degree of hepatic uptake whereas strongly anionized ones, e.g., phosphatidylserine-containing liposomes, PLGA have been considered to be taken up by liver non-parenchymal cells, via scavenger receptor mediated endocytosis of KC and SEC due to the direct recognition of their negative charge.

(d) Fc receptor
Fc receptors eliminate the soluble circulating immunoglobulin G immune complexes by receptor mediated endocytosis. Fc receptors exhibit delayed degradation of ligands than that internalized by scavenger expressed on SEC [119]. Fc receptors are unaltered till the necrotic foci are infiltrated, excessive injury with D-galactosamine in chronic inflammation. In such conditions, Fc receptors are minimized [120].

6.3.3.2 Hepatic Endothelial Cells

HSC express similar receptors as those found on KC, e.g., mannose receptors [11, 12], scavenger receptors internalizing advanced glycation end-products-Alb, maleylated bovine albumin, and fucoidan [121] and Fc receptors internalizing immune complexes of IgG and IgA. In addition, to these receptors, SEC possesses stabilin receptors and receptors for removal of extracellular matrix, e.g., laminin, hyaluronic receptor. Stabilin receptors are responsible for regulating the extracellular concentration of the matrices and their concentration in blood [122].

6.3.3.3 Hepatic Stellate Cells

HSC are involved in liver fibrosis or liver cirrhosis. Targeting HSC achieves decreased secretion of extracellular matrix [123].

(a) Phosphomannosyl receptor/Mannose 6 phosphate receptors
Phosphomannosyl receptor receptors are intracellularly located in the membranes of the endoplasmic reticulum, Golgi apparatus, and the lysosomes; only 10 % of the receptors are identified on the plasma membrane [124]. Delivery of newly synthesized lysosomal enzymes from the Golgi apparatus to lysosome in HSC requires the recognition of mannose 6-phosphate on these enzymes by a specific receptor—phosphomannosyl receptor/Mannose 6 phosphate receptors [125]. Targeting mannose 6 phosphate stimulates cytokines, converting growth factor β (TGF-β) stimulating production of collagen. Direct conjugation of mannose 6-phosphate with HPMA showed maximum uptake in diethyl nitrosamine induced liver fibrosis [54]. Albumin modified with mannose 6-phosphate selectively binds to hepatic stellate cells in fibrosis and accumulates up to 58 % of the injected dose after intravenous injection by endocytosis [126]. Inactivated hemagglutinating virus of Japan with a plasmid DNA tagged with luciferase has been targeted using liposomes decorated with albumin modified mannose-6-phosphate [127].

(b) Miscellaneous receptors
Retinol binding protein receptors (RBP) are also found unaltered in liver disorders and hence an important target in fibrosis [128]. A pro-drug Bexarotene (Targretin®) targets retinoic acid receptors in cancer. Cell surface integrins integrate with the matrix collagen type VI protein through Arg-Gly-Asp (RGD) dependent interactions via αvβ3 receptor. Intravenous injection of liposomes encapsulating siRNA responsible and conjugated with vitamin A suppressed collagen secretion as well as reduced fibrosis [129]. Covalent interaction of a cyclic octapeptide bearing "RGD" peptide to HSA increased the selective uptake by HSC [130]. Similarly cyclic peptide C*SRNLIDC* is recognized by platelet derived growth factor receptor (PDGF). RGD labeled liposomes have efficiently delivered interferon alpha-1b in fibrosis [131]. Conjugation to Human serum albumin incorporating an apoptotic drug led to accumulation on HSC [1].

Hepatitis C virus induces liver fibrosis and cirrhosis. Wang and coauthors have recently reported the presence of hepatitis C virus co-receptors responsible for promotion of liver fibrogenesis and engulfment of hepatocytes apoptotic bodies. In addition, many cytokine receptors, growth factor and transcription receptors, etc. are present on HSC, nevertheless with rare applications to liver targeting [39].

6.3.3.4 Hepatocytes

Hepatocytes are active targets in hepatic cellular carcinoma, hepatitis, steatohepatitis, genetic disorders, and metabolic disorders [132]. Hepatocytes receive the systemic circulation born substances after diffusion across the SEC separating blood

and hepatocytes. The particulates have foremost to pass through fenestrations of the sinusoid intravascular space generally of 100–200 nm [28, 133]. For active targeting in hepatocytes, introduction of steric stabilization, charge shielding techniques such as PEG layer reduce the opsonization and degradation in the lysosomes of KC.

(a) Asialoglycoprotein receptors (ASGP-R) or *N*-acetyl galactosamine receptor/ GlcNAc–R
ASGP-R clears off serum desialylated glycoproteins from the systemic circulation having non-reducing galactose or acetylgalactosamine residues, exposed at the end of their oligosaccharides, and through receptor mediated endocytosis [134]. The desialylated glycoproteins are subsequently processed through the liver lysosomes. The binding affinity of N-acetyl galactosamine residues to ASGP-R is 10- to 50-fold higher than ligands with only terminal galactose residues [135, 136]. A higher expression and density of 500,000 ASGP-R per cell has been reported on the basolateral side of hepatocytes [137] and on the side facing the sinusoidal area [138]. The ASGP-R possesses three Ca^{+2} dependent carbohydrate recognition domains and hence exhibits strong interaction—"cluster effect" with multivalent ligands (tri- or tetra-antennary *N*-linked glycans) [139]. Consequently, this results in lesser possibility of ligand escape towards other receptors [140, 141]. However, at higher surface density of galactose residues complete shift in uptake from hepatocytes to KC is observed due to ready recognition by the galactose receptors on Kupffer [142, 143]. Expression of ASGP-R in conditions like hepatocellular carcinoma is still of debate with reports of overexpression [144] as well as decreased expression [145]. Ligands ranging from asialofetuin soybean derived sterylglucoside, sito-G, arabinogalactan, pullulan, lactobionic acid to synthetically synthesized galactosylated ligand have been widely studied as ligands for ASGP-R. D'Souza et al. [146] and coauthors performed an in silico screening of various ligands (arabinogalactan, pullulan, and kappa carrageenan) for targeting ASGP-R and observed good correlation with liver distribution in healthy rats on intravenous administration of nanocarriers anchored with the ligands.

KC also express galactose receptor distinct from ASGP-R [147]. Functionally, both the receptors have affinity for galactose residue of lactose. However, specificity depends upon the degree of lactosylation. High substitution of lactosylated lipoprotein delivery targets the Kupffer cell (>300 lactose/LDL) despite being in minority, while at lower substitution (60 lactose /LDL); hepatocytes are targeted [148]. Lactosylated high density lipoprotein with diameter of 10 nm showed hepatocyte-specific targeting [142]. Galactose particle receptor has a high affinity for galactose, exposing particles with ligand size between 15 and 20 nm [145, 149, 150]. Liposomes with a lower degree of tri-antennary galactoside modification (5 % tri-antennary galactosides) were taken up by the ASGP-R on hepatocytes while those containing 50 % tri-antennary galactosides were taken up by KC [64, 143, 151]. Hence, an optimum balance of galactose density is desirable to prevent a shift in uptake from hepatocytes to galactose particle receptor on KC [142, 143].

(b) Glycyrrhizin (GL) and glycyrrhetinic acid (GA) receptors
Negishi et al. [152] demonstrated the presence of Glycyrrhizin and glycyrrhetinic acid receptors on the cellular membrane of hepatocytes. The binding sites for GA receptors surpass that of glycyrrhizin receptors [153]. GA is a metabolite of glycyrrhizin obtained from liquorice [154]. In vitro studies revealed 3.3-fold and 4.9-fold higher uptake for chitosan nanoparticles and poly(ethylene glycol)-b-poly(γ-benzyl L-glutamate) micelles, respectively modified with GA compared to unmodified nanoparticulates [155, 162]). Chemical conjugation of GA to nanoparticles increased internalization by liver cancer cells [156–161]. Derivatives of glycyrrhizin—30-stearyl glycyrrhizin [162]—also increased hepatic uptake. GA conjugated to hyaluronic acid has been suggested as a double targeting strategy for liver cancer by [163]. Recently the presence of GA receptors on HSC and tumor cells have also been reported [164].

(c) Integrin receptor
Vectors in Viral mediated delivery, viz., adenoviruses, retrovirus, hemagglutinating virus, lentiviral vectors innately are transduced through the adenoviral and integrin receptors [165–169]. The vector binds to the coxsackievirus-Ad receptor and is subsequently internalized by integrins. However, for hepatocyte transduction, this type of interaction between vector and CAR is not mandated [170, 171]. Uptake in KC causes degradation of genetic material. Immunogenicity and off-target effects have been improved by designing vectors derived from human immunodeficiency virus and pseudotyped with Sendai virus fusion protein F [166]. Though beta 1-integrin collagen receptors are reported to be present in hepatocytes, αVβ5 integrin receptor in KC have also been studied [172]. Integrin receptors are also associated with tumor blood vessels and are widely used for delivery of thrombolytic agents and anticancer drugs [173–175]. Specific targeting to hepatocytes through integrin is rare. Disruption of extracellular matrix related integrin signaling leads to termination of liver regeneration [101].

(d) Low-density lipoprotein receptor (LDLR)
Low-density lipoprotein receptor is an endocytotic type I transmembrane cell surface receptor and contributes to uptake of circulating cholesterol-rich LDL particles [176]. Uptake occurs via the clathrin-mediated receptor endocytosis system and is triggered by binding to the signaling proteins [177]. It maintains lipidic homeostasis as well as regulates fibrogenesis. Overexpression of LDLR is observed in non-alcoholic fatty liver disease and increases with advancement in fibrosis [178]. LDL metabolism has been associated with both hepatocytes and KC and follows saturation kinetics. Degradation of LDL is 18-fold higher in KC than in hepatocytes [179]. However, as in case of integrin receptors, LDLR are also known to be overexpressed in several tumors and have been widely studied for targeted delivery to malignant cells [180]. Hepatocyte specific delivery of disease-related genes using siRNAs has been cited [181, 182].

(e) Miscellaneous receptors
Targeting of apolipoprotein E (high density lipoprotein) and apolipoprotein A-1for delivery of siRNA and miRNA has been demonstrated via the class B type I scavenger receptor (SR-B1) [183, 184]. CD36 receptors are found to

retain inflammatory cells [185]. Lipid nanoparticles of acyclovir palmitate-recombinant HDL complex (~33 nm) revealed fourfold enhanced hepatic accumulation on intravenous injection [186, 187]. Scavenger receptors also have their existence on KC associated [118]. Transferrin receptor increases in patients with alcoholic liver diseases with excessive iron accumulation [188, 189] and is expressed in all nucleated cells [190].

Qin He incorporated hepatocyte specific AFP (α-fetoprotein) promoter to recombinant plasmid encapsulated in PLGA nanoparticles and achieved targeted delivery to hepatoma cells. AFP promoter exhibited specific activity only in cells containing α-fetoprotein [191]. Other techniques for hepatocyte targeting have been by conjugating with bile acids which exhibit hepatotropism to specific transport systems on the sinusoidal plasma membrane of hepatocyte [192]. A ligand activated nuclear receptor, Farnesoid X receptor, known to regulate lipid and glucose metabolism exhibits affinity to bile acids [193]. Other hepatocyte-specific transgene expression promoters are albumin, alpha 1-anti trypsin, enhanced transthyretin, etc. [194]. Receptors like cannabinoid receptor-1 though expressed on hepatocytes are also expressed in myofibroblasts, and adipose tissue and intestine bearing extrahepatic CB1 receptors [195]. Conditioning of KC with acetylated LDL or HDL increases the number of HDL receptors [196]. Recently receptors for mosquito-borne dengue viruses consisting of three proteins: heparan sulfate, the 37/67 kDa high-affinity laminin receptor, and prion protein have been reported [197, 198]. An association between the laminin receptor, a part ofDENV (Dengue virus) receptor and prion proteins was observed in HepG2 cells. Readers are directed to the following references for details [199, 200]. Glucagon like peptide receptors and δ opioid receptor have also been exploited [201–205]. The disposition of nanoparticulates in the liver is schematically depicted in Fig. 6.3 following intravenous injection.

6.3.3.5 Stem Cells

Cancer stem cells are generally resistant to chemotherapeutic drugs due to the presence of active transmembrane adenosine triphosphate-binding cassette (ABC) transporter family [206]. However, Wnt receptor, transmembrane Frizzled (Fzd) receptors, etc. are known to be stimulated on cancer stem cells [207, 208], while Notch and hedgehog signaling pathway inhibitors etc. can also serve as molecular target for cancer prevention by increasing the cell sensitivity to drugs and inhibiting drug efflux in both tumor cells and stem cells. Targeting to cancer stem cells has been proposed to be more efficient in eradicating and providing cure mainly to HCC [209, 210].

6.3.4 Stimuli Responsive Active Targeting

Drug release at specific sites can be triggered by external and/or internal stimuli. While magnetic [211], photo-irradiation, ultrasound [212], electric field, etc. are external stimuli [213, 214], internal stimuli include changes in pH [215], and

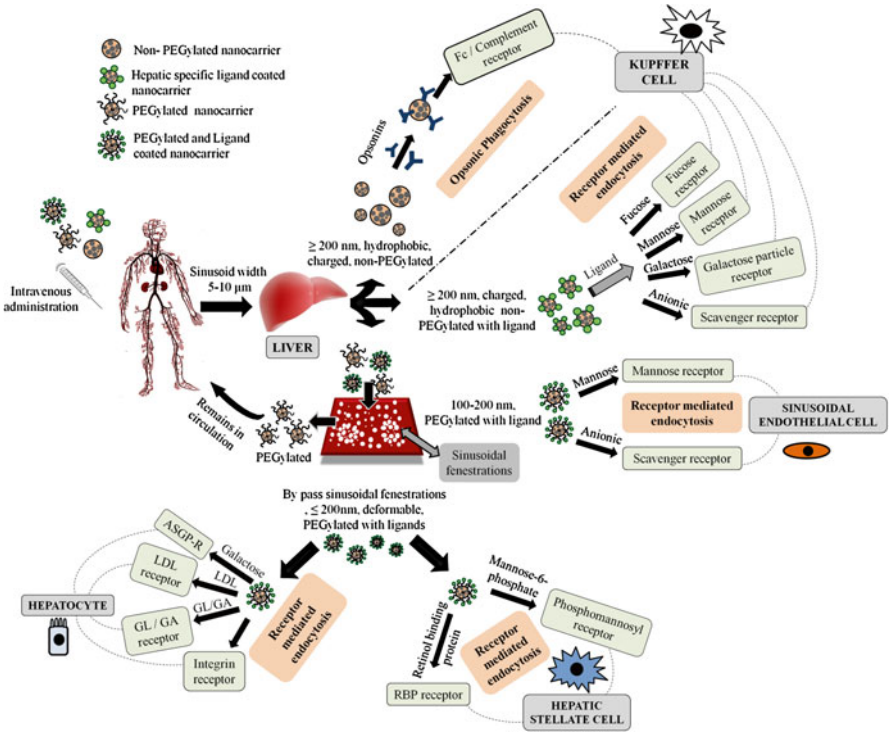

Fig. 6.3 Physiological fate of nanoparticles following intravenous administration (schematic)

temperature [216, 217] which can occur within organs during disorders or tumors. Dual stimuli—thermal and pH—responsive self-assembled structures of poly(*N-isopropylacrylamide*)-b-poly(L-histidine) were designed for controlled release of doxorubicin in liver carcinoma [218]. Thermodox®, doxorubicin containing PEGylated liposomes, is a temperature sensitive nanocarrier which releases drug only upon externally applied heat, i.e., radiofrequency ablation at the site of the tumor to raise the temperature above 39.5 C, or upon EPR-mediated passive tumor accumulation [219].

6.3.5 Antibody Mediated Active Targeting

Targeting with antibodies capable of recognizing and binding with affinity to antigens present on tumors as targeting strategy is widely explored [220, 221]. Immunoliposomes are widely exploited for delivery of anticancer agents [222]. ASGP-R single chain variable fragments on conjugation to immunotoxins exhibited an increased cytotoxicity in HepG2 and Huh7 cells compared to non-conjugated immunotoxin scFv fragments [223]. Interferon alpha genetically fused to a domain

antibody (dAb), an asialoglycoprotein receptor specific antibody, increased the in vivo targeting to liver [224]. Four Glypican-3 antibodies—GC33, YP7, HN3, MCX-1414—have been developed for cancer therapy and are under investigation [225]. Antibody mediated targeted delivery to CD133, an important surface marker of liver cancer stem cells, has shown good promise in cancer therapy [226]. Human recombinant single chain antibody (C1-3 scAb) to synaptophysin, a HSC selective expression, is being explored for targeting in anti-fibrotic therapy [227, 228].

6.4 Targeted Delivery Systems and Applications

Drugs administered orally or as injectables are efficiently removed from systemic circulation by KC due to first pass effect of drug causing metabolic transformation, detoxification, and excretion of drugs [229]. Nevertheless, KC largely contributes to uptake of particulate matter. Thus, drugs are precluded from reaching the desired cell type [230].

Covalent binding of therapeutically active drug to a liver targeting polymer improves liver targeting potential, circulation time and increases specificity [231]. Many polymers such as poly-lactic acid poly-glutamic acid (PLGA) have been galactosylated and have shown improved biodistribution over the conventional polymers [232]. Hydrolysis of drug from conjugates using enzymatic or environmental as triggers could also modify the drug kinetics. PEGylated conjugates are well reported for the success in treating various disorders including hepatitis C [233]. Protamine-asialofetuin lipoplexes contained asialofetuin as a natural targeting ligand to ASGP-R [234]. Oligonucleotide poly-L lysine polyplexes inhibited the expression of hepatitis B virus gene expression with increased hepatocyte uptake [235].

Nanotechnology has enabled systematic and site-specific delivery of drugs. Reviews on the same are abundant. Readers are directed to recent reviews [236]. Nanoparticles are high engineerable with integration of different physicochemical functionalities such as size, shape, hydrophobicity, etc. Further modification of surface properties such as charge, anchoring ligands, modulating ligand density for achieving selectivity with desired systemic effects has been explored. Exploitation of active and passive targeted strategy relies on the characteristics of nanocarriers. Nevertheless, administered drug achieve high and nonspecific accumulation in liver due to first pass effect for metabolism, while nanoparticles are efficiently removed from systemic circulation by the macrophages of RES especially liver (first-order targeting) [229] (Fig. 6.3). Though KC occupies only 40 % of liver cells, they are a major site of accumulation of nanoparticles (higher than parenchymal cells occupying 80 % and SEC occupying 6.3 %). Details of passive targeting to KC attributed to phagocytosis and factors contributing to phagocytic uptake are discussed earlier in Sect. 6.3.1.

Once passive targeting to KC occurs, disorders or conditions wherein accumulation of nanoparticles in hepatocytes or SEC is desirable are never achieved. Nevertheless, tailored nanoparticulates can be designed for efficient uptake by individual hepatic

cells. As mentioned in Sect. 3.2. Different strategies based on specificity of each receptor can be engineered on nanoparticles so as to achieve active targeting to different cells of liver. Another well-known technique is to administer a dose of blank particulates to saturate KC [67].

The advantages of each approach need to be weighed prior to designing nanoparticulates for active or passive targeting [237]. One such example in tumors and cancer is as follows: EPR permits inherent passive accumulation of nanoparticulates in tumor, thereby precluding the need for active targeting [238]. The nanoparticles evade the RES system either by manipulation of size, charge or by stealth coating and concurrently using an active targeting strategy enables specific cellular targeting. A combination of active targeting technique is also used to increase targeting efficiency. Hongfen Wei et al. prepared galactosylated docetaxel nanoparticles targeting hepatocytes in HCC combined with exposure to ultrasound to increase vascular permeability [239].

We present below the possible applications of targeted delivery to the liver for various liver afflictions in Table 6.2.

Nanoparticles targeting to liver using either passive or active targeting have been frequently used in the following disorders or conditions:

6.4.1 Hepatocellular Carcinoma or Hepatoma

Hepatocellular carcinoma (HCC) is a globally concerned disorder with a high mortality and accounts for 85 % of primary liver cancers [240, 241]. Physiology of the liver is altered and could be exploited for passive targeting [242]. As observed in other tumors, HCC also exhibit leaky vasculature with discontinuous endothelial cell lining with pores (600–800 nm and at times up to 2 μm) [243–245]. Nanoparticulates up to 400 nm can easily extravasate through the leaky vasculature and result in increased drug concentration in the tumor [245, 246]. Macromolecules larger than 40 kDa and smaller than the fenestrations can easily accumulate in tumor tissue [244]. This condition is also a boon for active targeting using pH dependent release. Tumor cells show an increased glycolysis causing an acidic tumor microenvironment [247]. The acidic microenvironment of tumors due to increased glycolysis also permits pH triggered drug release from liposomes. Active targeting of hepatocellular carcinoma has been achieved mainly by targeting the ASGP-R [248], retinoic acid receptor [249], glycyrrhetinic receptors [250], LDL receptors [251], etc. Most of the targeting strategies for treatment of HCC are based on binding to ASGP-Rs utilizing galactose as targeting agents [252]. But to date, very few nanocarriers have been developed [187].

SMANCS, a conjugate of Poly(styrene maleic acid)—SMA—and the protein antitumor agent neocarzinostatin—NCS—in Lipiodol—lipid contrast agent, an oily formulation has been selectively used in the treatment of HCC in Japan since 1993 [253, 254]. It is devoid of side effects caused by conventional chemotherapeutic agents and suitable for X-ray computed tomography [255].

Table 6.2 Receptors, ligands, and nanocarriers for liver targeted delivery

Diseases	Nanocarriers	Ligand	Receptor	Reference
Hepatocellular carcinoma	Albumin–chitosan nanoparticles	Retinoic acid	Retinol binding protein receptor on HSC	[249]
	DOX loaded in glycyrrhetinic acid tagged chitosan–PEG nanoparticles	Glycyrrhetinic acid	Glycyrrhetinic acid receptors on hepatocytes	[157, 160]
	IL-6-receptor and/or immunoglobulin A binding protein	Pre-S1	IL-6-receptor on hepatocytes	[317]
	Doxorubicin loaded in glycyrrhetinic acid tagged alginate nanoparticles	Glycyrrhetinic acid	Glycyrrhetinic acid receptors on hepatocytes	[250]
	Doxorubicin delivery by chitosan-galactosylated modified polymer microbubbles	Galactose	Asialoglycoprotein receptor	[248]
	G5.0 PPI dendrimers to construct LDL- and HDL-conjugated dendrimeric nanoconstructs	LDL- and HDL	LDLR and HDLR	[251]
	Sulfated chitosan	Glycyrrhetinic acid	Glycyrrhetinic acid on hepatocytes	[159]
	Polymer conjugates (Phase I clinical trial)	Galactoseamine	ASGP-R on hepatocytes	[145]
	Oridonin loaded galactosylated bovine serum albumin nanoparticle	Galactosylated bovine serum albumin	ASGP-r on hepatocytes	[318]
	Trans-resveratrol loaded chitosan nanoparticles	Biotin and avidin	Biotin and avidin receptors specifically on hepatic cancer cells	[319]
Hepatic fibrosis	DOX loaded Poly(benzyl malate) bearing biotin	Biotin	Biotin on hepatic cancer cells	
	Sodium ferulate loaded on Albumin nanoparticles		Mannose 6-phosphate in hepatic stellate cells	[164]
	Liposomes encapsulating interferon alpha-1b	Cyclic RGD	Type VI collagen receptor on HSC	[130, 131, 320, 321]
	15-deoxy-delta12,14-prostaglandin J2	PDGF	PDGF receptor on HSC	[322]
	Hemagglutinin virus of Japan in liposomes	Mannose 6-phosphate	Mannose 6-phosphate in hepatic stellate cells	[127]
	Liposome containing siRNA against gp46	Vitamin A	Retinol binding protein receptor	[129]
	Liposomes containing quercetin	Galactose	ASGP-R	[323]

(continued)

Table 6.2 (continued)

Diseases	Nanocarriers	Ligand	Receptor	Reference
Genetic diseases	*N*-acetylgalactosamine functionalized polyethylene glycol -b-Poly(episol-caprolactone) micelles	siRNA targeting apolipoprotein B	ASGP-R on Hepatocytes	[328]
	Nanoassociates of DNA and hyaluronic acid coupled to polymer	Gene delivery	hyaluronic receptor on HSC	[324, 325]
	PLGA nanoparticles modified with mannan PE for gene delivery	Mannan-based PE-grafted ligands	Mannan receptor	[93]
	Galactosylated Polyethylenimine-graft-poly(ethylene glycol) conjugated with gene	Galactose	ASGP-R	[326]
	Mannose Polyethylenimine DNA	Mannose	Mannose receptor	[327]
	Galactosylated micelles for ribavirin delivery	Galactose	ASGP-R	[268]
	Doxorubicin delivered through cross-linked micelles	Galactose	ASGP-R	[328, 329]
	Naked pCMV DNA entrapped in PLGA nanoparticles	Asialofetuin	ASGP-R	[330]
Infectious diseases	Lactose modified LDL, Tri-gal-chol-LDL Galactose coated dendrimer of primaquine phosphate Apolipoprotein E anchored chylomicron of primaquine phosphate Nanoemulsion of primaquine phosphate	Galactosylated/ lactosylated low density lipoprotein Galactose Apolipoprotein E Apolipoprotein E	LDL on KC ASGP-R Apolipoprotein E Receptor Apolipoprotein E Receptor	[176, 264, 331, 332]
	Ribavirin linked nanoparticles by self-assembly of amphiphilic copolymer	Lactose	ASGP-R on hepatocytes	[320, 321]

6.4.2 Infectious Diseases

The liver being home for many transport machineries and almost 80 % of the macrophage population, foreign bodies and large therapeutic molecules (Molecular weight ~50 kDa) achieve high hepatic concentration on administration [256]. Nano drug delivery systems also render high uptake of particulates by the macrophagic KC [246, 257]. This physiological phenomenon could be an advantage for treatment of macrophage related infections (Leishmaniasis, AIDS, Brucellosis, Listeriosis, Mycobacteria, and Salmonella infections).

In parasitic infections like malaria, the sporozoites of Plasmodium selectively infect erythrocytes and human hepatocytes. This erythrocytic stage of Plasmodium causes increased gametocyte production and subsequently sequestration in systemic circulation [258]. Though the size of sporozoites exceeds the size of fenestrations, the sporozoites from blood sequester hepatocytes using proteoglycans by squeezing through the endothelial fenestration [259]. Targeted delivery of Primaquine to the liver has been evaluated following intravenous administration of liposomes [260, 261] and gelatin and albumin nanoparticles [262]. Preferential delivery of Primaquine to the hepatocytes was achieved using an artificial chylomicron emulsion [263] and galactosylated liposomes [139]. Dendrimeric nanoparticles of PQ coated with galactose, a ligand for the ASGPR receptor on hepatocytes, also favored high accumulation of PQ in the hepatocytes [264].

6.4.3 Nucleic Acid Delivery

Chemotherapies at times cannot address issues which have caused specific mutations or alterations, and hence genetic delivery becomes mandatory. However, nucleic acids possesses large size, anionic charge repulsion, hydrophilic highly charged and possesses short half-life due to nucleases and metabolic nature of liver violating the Lipinski's rule of 5 [265]. Cellular targeting of genetic material is often construed a herculean task. The journey begins from protection of the genetic material in systemic circulation. Cationic liposomes and nanoparticles conceal the genetic material while facilitating cellular uptake [72]. Further, cationic polymers exhibit strong buffering capacity between pH 5–7 causing osmotic swelling and finally vacuole disruption releasing the genetic material into cytoplasm [266]. Genetic transfer specifically to hepatocytes by incorporation of hepatocyte-specific promoters (albumin, alpha 1-anti trypsin, or enhanced transthyretin, α-fetoprotein, etc.) in lentiviruses (retroviral mediated genetic delivery) is reported. This reduces the expression on non-parenchymal cells [194]. High efficient transfer of siRNA using polyconjugates [267], polymeric micelles [268], and self-assembled amphiphilic cationic copolymers [269] have been studied. Gene targeting of human Factor FVIII using gamma retroviral vectors to hepatocytes in hemophilia A has been prompted for Phase I clinical trials in patients. Synthesis of F factors occurs primarily in the liver and is supplied to the blood. Majority of the patients exhibited good tolerance to the

treatment. Targeting of these genes to antigen presenting cells increases the immunity and decreases long term expression. Hence, a hepatocyte specific promoter is mandated. Micro-RNAs especially for liver diseases, inflammation, and cirrhosis are under investigation [270].

The inability of sinusoids to form a barrier for proteins has been long known. Hence, viral vector mediated gene delivery successfully exhibit its expression in hepatocytes rather than non-parenchymal cells. Also, the genetic material tends to degrade in the lysosomes of KC. Exploitation of protein for hepatocyte targeting has thus been widely studied. Giri explored this strategy for delivery of interferon-α using cationic PLGA nanoparticles with hepatitis B surface antigen (HBsAg) adsorbed onto its surface. The author proposed the system as an artificial viral vector [271]. Similarly, PEGylated interferon (Pegasys, PEG-Intron) has been successfully targeted to hepatocytes in hepatitis by passive targeting [272]. The stealth property imparted by PEGylation increases the circulation time thereby favoring high uptake. Since the uptake is not attributed to specific receptors, uptake in non-desired sites has also been observed [273]. Jung and coauthors designed core shell nanoparticles with a hollow core and a shell made of HBV envelope (bio-nanocapsule) which had pre-S1 peptide as a ligand for hepatocytes. The bio-nanocapsule was conjugated to liposomes for peptide delivery [187, 274].

Efficiency of gene delivery can also be enhanced by temporarily depleting the KC. Depletion of KC can be achieved by administration of clodronate liposomes. The technique is however risky as the reappearance of KC would take up to 1 week [275]. Depressed blood flow, endotoxemia, and bacteremia are also associated with decreasing clearance activity in Kupffer cell.

6.5 Imaging and Diagnosis

Over the decades, considerable advancement has evolved in diagnostic detection of various liver disorders. Common techniques for detection of HCC are quantifying the serum αfetoprotein or magnetic resonance imaging. The most common being the later, except in tumors less than 2 cm [276]. Radiopharmaceuticals containing galactose, lactose, or *N*-acetyl galactosamine recognizing ASGP-R on hepatocytes are used as nuclear imaging radiopharmaceuticals targeting hepatocytes [277].

Table 6.3 summarizes some of the major approaches for diagnosis of liver conditions.

6.6 Future Directions

Nanocarriers for targeted delivery in liver afflictions are in clinical investigation for therapy and diagnosis (Table 6.4). Polyisohexylcyanoacrylate nanoparticles encapsulating doxorubicin was the first nanoparticulate to enter in clinical trials for HCC. However, associated pulmonary adverse effects resulted in suspension of the

Table 6.3 Diagnostic interventions for liver targeted delivery

Diagnosis	Ligand	Receptor	Reference
Bioimaging of quantum dots	Hyaluronic acid derivative	Hyaluronic acid receptors	[333]
	D-galactose	ASGP-R	[334]
Fluorescence imaging	Glypican-3	Antibody mediated targeting specific for HCC	[335]
Radioactivity	99mTc hydrazino nicotinamide-galactosylated chitosan	ASGP-R	[336]
	99mTc- galactosylated chitosan	ASGP-R	[326]
	99mTc-gold nanoparticles capped with HYNIC-peptide/mannose	Mannose receptor	[337]
MRI	poly(propylene imine) dendrimers composed of GdDTPA and cyclic NGR	Cyclic NGR (similar to RGD) binding to collagen type IV protein	[338]
	PLA-PEG/Gd-DTPA	Passive accumulation	[339, 347]
	PLA-PEG-NH2 immobilized with FITC through biotin–avidin system and anti-alpha-fetoprotein	Biotin avidin receptors on hepatocytes affected with cancer	[339]
	LDLR-targeted amphiphilic gadolinium (Gd)-diethylenetriaminepentaacetic acid chelates	LDLR	
	Galactosylated manganese ferrite nanoparticles	ASGP-R	[340]
	Mannan-coated superparamagnetic iron oxide nanoparticles	Mannose receptors	[341]
	Gadolinium labeled LDL nanoparticles	LDL receptor	[342]
	Gadolinium labeled cholesterol-HDL nanoparticles	HDL receptor	[180]

Table 6.4 Clinical trial and commercialization status of liver targeted delivery system

Clinical trials	Brand name	Phase in study	Reference
Doxorubicin loaded poly(alkyl cyanoacrylate) nanoparticles	Transdrug for HCC	Phase II and III	[343]
Hepatic arterial infusion of nanoparticle albumin-bound paclitaxel	–	Phase I	[344]
HPMA bearing doxorubicin with galactosamine (PK2)		Phase I	[145]
PEG–arginine deiminase (i.v.)	Hepacid by Phoenix for HCC	Phase I/II	[231]
Virosomal hepatitis vaccine (Liposomal IRIV)	Epaxal Berna Hepatitis A	Marketed by Berna Biotech (Bern, Switzerland)	[345]
PEG–alpha-interferon 2a	Pegasys for Hepatitis C	Nektar (San Carlos, CA, USA), Hoffmann-La Roche (Basel)	[345]
PEG–interferon 2b	PEG-Intron for Hepatitis C	Enzon, schering-plough	[345]
Iron nanoparticles for imaging liver tumors	Resovist	Schering (Berlin)	[345]
Iron nanoparticles for imaging liver tumors	Feridex/Endorem	Advanced magnetics (Cambridge, MA, USA), Guerbet (Roissy, France)	[345]

Phase II study. Other unexplored areas for targeting parasites invading the liver include amoebic liver abscesses, hydatid cyst of the liver, fluke diseases, hemophilia, type I tyrosinemia, Wilson disease, etc. A recent upcoming area is the pharmacological modulation of the phenomenon autophagy for therapy of liver disorders. Autophagy is a process of lysosomal degradation of bulk cytoplasm or damaged organelles [278]. Improved therapeutic and diagnostic efforts have changed the status of hepatic cancer from dreadful to at least a treatable disease.

References

1. Li L, Wang H, Ong ZY, Xu K, Ee PLR, Zheng S, Hedrick JL, Yang YY (2010) Polymer- and lipid-based nanoparticle therapeutics for the treatment of liver diseases. Nano Today 5:296–312
2. Sawey ET, Chanrion M, Cai C, Wu G, Zhang J, Zender L, Zhao A, Busuttil RW, Yee H, Stein L, French DM, Finn RS, Lowe SW, Powers S (2011) Identification of a therapeutic strategy targeting amplified FGF19 in liver cancer by oncogenomic screening. Cancer Cell 19:347–358
3. Santi V, Buccione D, Di Micoli A, Fatti G, Frigerio M, Farinati F, Poggio PD, Rapaccini G, Di Nolfo MA, Benvegnù L, Zoli M, Borzio F, Giannini EG, Caturelli E, Chiaramonte M, Bernardi M, Trevisani F (2012) The changing scenario of hepatocellular carcinoma over the last two decades in Italy. J Hepatol 56:397–405
4. Rustgi VK (1987) Epidemiology of hepatocellular carcinoma. Gastroenterol Clin North Am 16:545–551
5. Avila MA, Berasain C, Sangro B, Prieto J (2006) New therapies for hepatocellular carcinoma. Oncogene 25(27):3866–3884
6. Popovic Z, Liu W, Chauhan VP, Lee J, Wong C, Greytak AB, Insin N, Nocera DG, Fukumura D, Bawendi MG (2010) A nanoparticle size series for in vivo fluorescence imaging. Angew Chem Int Ed Engl 49:8649–8652
7. De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJ, Geertsma RE (2008) Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29(12):1912–1919
8. Suzuki A (2013) Artificial induction and disease-related conversion of the hepatic fate. Curr Opin Genet Dev 23:579–584
9. Orrego H, Medline A, Blendis LM, Rankin JG, Kreaden DA (1979) Collagenisation of the disse space in alcoholic liver disease. Gut 20:673–679
10. Thomas P (2012) Kupffer cells. Encyclopedia of Cancer 1963–1965
11. Elvevold K, Simon-Santamaria J, Hasvold H, McCourt P, Smedsrod B, Sorensen KK (2008) Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity. Hepatology 48(6):2007–2015
12. Elvevold K, Smedsrod B, Martinez I (2008) The liver sinusoidal endothelial cell: a cell type of controversial and confusing identity. Am J Physiol-Gastrointest Liver Physiol 294: G391–G400
13. Saxena R, Theise ND, Crawford JM (1999) Microanatomy of the human liver-exploring the hidden interfaces. Hepatology 30:1339–1346
14. Fausto N, Campbell JS (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120:117–130
15. Bilzer M, Roggel F, Gerbes AL (2006) Role of Kupffer cells in host defense and liver disease. Liver Int 26:1175–1186
16. Hilmer SN, Cogger VC, Le Couteur DG (2007) Basal activity of Kupffer cells increases with old age. J Gerontol A Biol Sci Med Sci 62(9):973–978

17. Bouwens L, Baekeland M, Wisse E (1984) Importance of local proliferation in the expanding Kupffer cell population of rat liver after Zymosan stimulation and partial hepatectomy. Hepatology 4:213–219
18. Kolios G, Valatas V, Kouroumalis E (2006) Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol 12(46):7413–7420
19. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, Scott DK, O'Doherty RM (2010) Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 59:347–357
20. Traeger T, Mikulcak M, Eipel C, Abshagen K, Diedrich S, Heidecke CD, Maier S, Vollmar B (2010) Kupffer cell depletion reduces hepatic inflammation and apoptosis but decreases survival in abdominal sepsis. Eur J Gastroenterol Hepatol 22:1039–1049
21. Sakai T, Liu L, Teng X, Ishimaru N, Mukai-Sakai R, Tran NH, Kim SM, Sano N, Hayashi Y, Kaji R, Fukui K (2010) Inflammatory disease and cancer with a decrease in Kupffer cell numbers in Nucling-knockout mice. Int J Cancer 126(5):1079–1094
22. Loegering DJ (1986) Kupffer cell complement receptor clearance function and host defense. Circ Shock 20(4):321–333
23. Kitani H, Takenouchi T, Sato M, Yoshioka M, Yamanaka N (2010) A novel isolation method for macrophage-likecells from mixed primary cultures of adult rat liver cells. J Immunol Methods 360:47–55
24. Kmiec Z (2001) Cooperation of liver cells in health and disease. Adv Anat Embryol Cell Biol 161:III–XIII, 1–151
25. Bayo NLG, Izquierdo MA, Sirovich I, Rooijen NV, Beelen RHJ, Meijer S (2003) Role of kupffer cells in arresting circulating tumor cells and controlling metastatic growth in the liver. Hepatology 23:1224–1231
26. Willekens FLA, Werre JM, Kruijt JK, Roerdinkholder-Stoelwinder B, Groenen-Döpp YA, van den Bos AG, Bosman GJ, van Berkel TJ (2005) Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. Blood 105(5):2141–2145
27. Ruf H, Gould BJ (1999) Size distributions of chylomicrons from human lymph from dynamic light scattering measurements. Eur Biophys J 28(1):1–11
28. Jacobs F, Wisse E, De Geest B (2010) The role of liver sinusoidal cells in hepatocyte—directed gene transfer. Am J Pathol 176:14–21
29. Donald KJ, Tennent RJ (1975) The relative roles of platelets and macrophages in clearing particles from the blood; the value of carbon clearance as a measure of reticuloendothelial phagocytosis. J Pathol 117:235–245
30. Foley EM, Esko JD (2010) Hepatic heparan sulfate proteoglycans and endocytic clearance of triglyceride-rich lipoproteins. Prog Mol Biol Transl Sci 93:213–233
31. Malovic I, Sorensen KK, Elvevold KH, Nedredal GI, Paulsen S, Erofeev AV, Smedsrod BH, McCourt PA (2007) The mannose receptor on murine liver sinusoidal endothelial cells is the main denatured collagen clearance receptor. Hepatology 45(6):1454–1461
32. Benten D, Follenzi A, Bhargava KK, Kumaran V, Palestro CJ, Gupta S (2005) Hepatic targeting of transplanted liver sinusoidal endothelial cells in intact mice. Hepatology 42(1):140–148
33. DeLeve LD (2011) Vascular liver disease and the liver sinusoidal endothelial cell. In: DeLeve LD, Garcia-Tsao G (eds) Vascular liver disease: mechanisms and management. LLC, Springer Science Business Media, New York, pp 25–40
34. Vogel S, Piantedosi R, Frank J, Lalazar A, Rockey DC, Friedman SL, Blaner WS (2000) An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. J Lipid Res 41:882–893
35. Pinzani M, Marra F (2001) Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis 21(3):397–416
36. Geerts A (2001) History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin Liver Dis 21(3):311–335
37. Winau F, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, Modlin RL, Liblau RS, Gressner AM, Kaufmann SH (2007) Ito cells are liver-resident antigen-presenting cells for activating T cell responses. Immunity 26(1):117–129

38. Moreira RK (2007) Hepatic stellate cells and liver fibrosis. Arch Pathol Lab Med 131(11): 1728–1734
39. Friedman SL (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 88(1):125–172
40. Dancygier H (2010) Microscopic anatomy. In: Dancygier H (ed) Clinical hepatology: principles and practice of hepatobiliary diseases, vol 1. Springer, Berlin, pp 15–52
41. Luo DZ, Vermijlen D, Ahishali B, Triantis V, Plakoutsi G, Braet F, Vanderkerken K, M 5076 metastasis-bearing mice. Canc Chemother Pharmacol 26:122–126
42. Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E, Braet F, Wisse E (2004) Pit cells exclusively kill P815 tumor cells by the perforin/granzyme pathway. Comp Hepatol 3(Suppl 1):S58
43. Wang SR, Renaud G, Infante J, Catala D, Infante R (1985) Isolation of rat hepatocytes with EDTA and their metabolic functions in primary culture. In Vitro Cell Dev Biol 21(9):526–530
44. Sabine C, Perret C (2011) Liver Zonation. In: Monga SPS (ed) Molecular pathology of liver diseases, molecular pathology library 5. Springer Science + Business Media, LLC, New York, pp 7–16
45. Kan NG, Junghans D, Belmonte JCI (2009) Compensatory growth mechanisms regulated by BMP and FGF signaling mediate liver regeneration in zebrafish after partial hepatectomy. FASEB J 23(10):3518–3525
46. Donthamsetty S, Bhave VS, Kliment CS, Bowen WC, Mars WM, Bell AW, Stewart RE, Orr A, Wu C, Michalopoulos GK (2011) Excessive hepatomegaly of mice with hepatocyte-targeted elimination of integrin linked kinase following treatment with 1,4-bis (2-(3,5-dichaloropyridyloxy)) benzene. Hepatology 53(2):587–595
47. Chavez PR, Lian F, Chung J, Liu C, Paiva SA, Seitz HK, Wang XD (2011) Long-term ethanol consumption promotes hepatic tumorigenesis but impairs normal hepatocyte proliferation in rats. J Nutr 141(6):1049–1055
48. Liu WH, Zhao YS, Gao SY, Li SD, Cao J, Zhang KQ, Zou CG (2010) Hepatocyte proliferation during liver regeneration is impaired in mice with methionine diet-induced hyperhomocysteinemia. Am J Pathol 177(5):2357–2365
49. Lemaigre F, Zaret KS (2004) Liver development update: new embryo models, cell lineage control, and morphogenesis. Curr Opin Genet Dev 14(5):582–590
50. Ramaa CS, Tilekar KN, Patil VM (2010) Liver cancer: different approaches for targeting. Int J PharmTech Res 2:834–842
51. Sell S (2003) The hepatocyte: heterogeneity and plasticity of liver cells. Int J Biochem Cell Biol 35:267–271
52. Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, Nakauchi H, Taniguchi H (2006) Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 44:240–251
53. Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T (2006) Moriwaki H (2006) Characterization of CD133+ hepatocellular carcinoma cells ascancer stem/progenitor cells. Biochem Biophys Res Commun 351(4):820–824
54. Yang ZF, Ngai P, Ho DW, Yu WC, Ng MN, Lau CK, Li ML, Tam KH, Lam CT, Poon RT, Fan ST (2008) Identification of local and circulating cancer stem cells in human liver cancer. Hepatology 47:919–928
55. Dai H, Jiang X, Tan GC, Chen Y, Torbenson M, Leong KW, Mao HQ (2006) Chitosan-DNA nanoparticles delivered by intrabiliary infusion enhance liver-targeted gene delivery. Int J Nanomedicine 1(4):507–522
56. Gao L, Lisi Xie L, Long X, Wang Z, He CY, Chen ZY, Zhang L, Nan X, Lei H, Liu X, Liu G, Lu J, Qiu B (2013) Efficacy of MRI visible iron oxide nanoparticles in delivering minicircle DNA into liver via intrabiliary infusion. Biomaterials 34(14):3688–3696
57. Jiang X, Ren Y, Williford JM, Li Z, Mao HQ (2013) Liver-targeted gene delivery through retrograde intrabiliary infusion. Methods Mol Biol 948:275–284
58. Fumoto S, Kawakami S, Hashida M, Nishida K (2013) Targeted gene delivery: importance of administration routes. In Wei M, Good D (eds) Novel gene therapy approaches ISBN: 978-953-51-0966-2, InTech, DOI: 10.5772/54741

59. Allen TM, Murray L, MacKeigan S, Shah M (1984) Chronic liposome administration in mice: effects on reticuloendothelial function and tissue distribution. J Pharmacol Exp Ther 229:267–275
60. Hsu MJ, Juliano RL (1982) Interactions of liposomes with the reticuloendothelial system. Non-specific and receptor-mediated uptake of liposomes by mouse peritoneal macrophages. Biochim Biophys Acta 720:411–419
61. Nie S (2010) Understanding and overcoming major barriers in cancer nanomedicine. Nanomedicine (Lond) 5(4):523–528
62. Bachmann MF, Jennings GT (2010) Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol 10:787–796
63. Brannon-Peppas L, Blanchette JO (2004) Review Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev 56(11):1649–1659
64. Rensen PCN, Sliedregt LAJM, Ferns M, Kieviet E, van Rossenberg SMW, van Leeuwen SH, van Berkel TJC, Biessen EAL (2001) Determination of the upper size limit for uptake and processing of ligands by the asialoglycoprotein receptor on hepatocytes in vitro and in vivo. J Biol Chem 276(40):37577–37584
65. Petros RA, DeSimone JM (2010) Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discov 9:615–627
66. Venturoli D, Rippe B (2005) Ficoll and dextran vs. globular proteins as probes for testing glomerular permselectivity: effects of molecular size, shape, charge, and deformability. Am J Physiol Renal Physiol 288:F605–F613
67. Bertrand N, Leroux JC (2012) The journey of a drug-carrier in the body: an anatomo-physiological perspective. J Control Release 161:152–163
68. Cannon GJ, Swanson JA (1992) The macrophage capacity for phagocytosis. J Cell Sci 101:907–913
69. Liu D, Mori A, Huang L (1991) Large liposomes containing ganglioside GM1 accumulate effectively in spleen. Biochim Biophys Acta 1066:159–165
70. Porter CJH, Moghimi SM, Illum L, Davis SS (1992) The polyoxyethylene polyoxypropylene block copolymer Poloxamer-407 selectively redirects intravenously injected microspheres to sinusoidal endothelial-cells of rabbit bone-marrow. FEBS Lett 305:62–66
71. Vasir JK, Reddy MK, Labhasetwar VD (2005) Nanosystems in drug targeting: opportunities and challenges. Curr Nanosci 1:47–64
72. Angart P, Vocelle D, Chan C, Walton SP (2013) Design of siRNA therapeutics from the molecular scale. Pharmaceuticals (Basel) 6:440–468
73. He C, Hu Y, Yin L, Tang C, Yin C (2010) Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials 31: 3657–3666
74. Li SD, Huang L (2010) Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. J Control Release 145(3):178–181
75. Alexis F, Pridgen E, Molnar LK, Farokhzad OC (2008) Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharm 5(4):505–515
76. Socha M, Lamprecht AE, Ghazouani F, Emond E, Maincent P, Barré J, Hoffman M, Ubrich N (2008) Increase in the vascular residence time of propranolol-loaded nanoparticles coated with heparin. J Nanosci Nanotechnol 8(5):2369–2376
77. Wu J, Liu L, Yen RD, Catana A, Nantz MH, Zern MA (2004) Liposome-mediatedextracellular superoxide dismutase gene delivery protects against acuteliver injury in mice. Hepatology 40:195–204
78. Park JH, Cho HJ, Yoon HY, Yoon IS, Ko SH, Shim JS, Cho JH, Park JH, Kim K, Kwon IC, Kim DD (2014) Hyaluronic acid derivative-coated nanohybrid liposomes for cancer imaging and drug delivery. J Control Release 174:98–108
79. Carmona S, Jorgensen MR, Kolli S, Crowther C, Salazar FH, MarionPL FM, Natori Y, Thanou M, Arbuthnot P, Miller AD et al (2009) Controlling HBV replication in vivo by intravenous administration of triggered PEGylated siRNA-nanoparticles. Mol Pharm 6:706–717
80. Champion JA, Katare YK, Mitragotri S (2007) Particle shape: a new design parameter for micro- and nanoscale drug delivery carriers. J Control Release 121(1–2):3–9

81. Champion JA, Mitragotri S (2006) Role of target geometry in phagocytosis. Proc Natl Acad Sci U S A 103:4930–4934
82. Devarajan PV, Jindal AB, Patil RR, Mulla F, Gaikwad RV, Samad A (2010) Particle shape: a new design parameter for passive targeting in splenotropic drug delivery. J Pharm Sci 99(6):2576–2581
83. Decuzzi P, Pasqualini R, Arap W, Ferrari M (2009) Intravascular delivery of particulate systems: does geometry really matter? Pharm Res 26(1):235–243
84. Qiu Y, Liu Y, Wang L, Xu L, Bai R, Ji Y, Wu X, Zhao Y, Li Y, Chen C (2010) Surface chemistry and aspect ratio mediated cellular uptake of Au nanorods. Biomaterials 31(30): 7606–7619
85. Banquy X, Suarez F, Argaw A, Rabanel JM, Grutter P, Bouchard JF, Hildgen P, Giasson S (2009) Effect of mechanical properties of hydrogel nanoparticles on macrophage cell uptake. Soft Matter 5:3984–3991
86. Romero EL, Morilla MJ, Regts J, Koning GA, Scherphof GL (1999) On the mechanism of hepatic transendothelial passage of large liposomes. FEBS Lett 448:193
87. Beningo KA, YL (2002) Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target. J Cell Sci 115:849–856
88. Protzer U, Maini MK, Knolle PA (2012) Living in the liver: hepatic infections. Nat Rev Immunol 12:201–213
89. Kapse SV, Gaikwad RV, Samad A, Devarajan PV (2012) Self nanoprecipitating preconcentrate of tamoxifen citrate for enhanced bioavailability. Int J Pharm 429(1–2):104–112
90. Galvin P, Thompson D, Ryan KB, McCarthy A, Moore AC, Burke CS, Dyson M, MacCraith BD, Gunko YK, Byrne MT, Volkov Y, Keely C, Keehan E, Howe M, Duffy C, MacLoughlin R (2012) Nanoparticle-based drug delivery: case studies for cancer and cardiovascular applications. Cell Mol Life Sci 69:389–404
91. Hillaireau H, Couvreur P (2009) Nanocarriers' entry into the cell: relevance to drug delivery. Cell Mol Life Sci 66:2873–2896
92. Shah NB, Vercellotti GM, Bischof JC (2012) Blood—nanoparticle interactions and in vivo biodistribution: impact of surface PEG and ligand properties. Mol Pharm 9:2146–2155
93. Kong F, Ge L, Liu X, Huang N, Zhou F (2012) Mannan-modified PLGA nanoparticles for targeted gene delivery. Int J Photoenerg Article ID 926754, 7p. doi:10.1155/2012/926754
94. Ganta S, Devalapally H, Shahiwala A, Amiji M (2008) A review of stimuli-responsive nanocarriers for drug and gene delivery. J Control Release 126(3):187–204
95. Muro S (2012) Challenges in design and characterization of ligand-targeted drug delivery systems. J Control Release 164:125–137
96. Torchilin VP (2000) Drug targeting. Eur J Pharm Sci 11(Suppl. 2):S81–91
97. Lammers T, Kiessling F, Hennink WE, Storm G (2012) Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress. J Control Release 161(2):175–187
98. Heath TD, Fraley RT, Bentz J, Voss EWJ, Herron JN, Papahadjopoulos D (1984) Antibody-directed liposomes. Determination of affinity constants for soluble and liposome bound anti-fluorescein. Biochim Biophys Acta 770:148–158
99. Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422:37–44
100. Sahay G, Alakhova DY, Kabanov AV (2010) Endocytosis of nanomedicines. J Control Release 145:182–195
101. Xu C, Yang Y, Yang J, Chen X, Wang G (2012) Analysis of the role of the integrin signaling pathway in hepatocytes during rat liver regeneration. Cell Mol Biol Lett 17(2):274–288
102. Roger E, Lagarce F, Garcion E, Benoit JP (2010) Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery. Nanomedicine (Lond) 5:287–306
103. Albanese A, Tang PS, Chan WC (2012) The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu Rev Biomed Eng 14:1–16
104. Beljaars L, Poelstra K, Molema G, Meijer DKF (1998) Targeting of sugar- and charge modified albumins to fibrotic rat livers: the accessibility of hepatic cells after chronic bile duct ligation. J Hepatol 29:579–588

105. Higuchi Y, Nishikawa M, Kawakami S, Yamashita F, Hashida M (2004) Uptake characteristics of mannosylated and fucosylated bovine serum albumin in primary cultured rat sinusoidal endothelial cells and Kupffer cells. Int J Pharm 287:147–154
106. Melgert BN, Olinga P, Jack VK, Molema G, Meijer DKF, Poelstra K (2000) Dexamethasone coupled to albumin is selectively taken up by rat nonparenchymal liver cells and attenuates LPS-induced activation of hepatic cells. J Hepatol 32:603–611
107. Ishimoto N, Nemoto T, Nagayoshi F, Yamashita MH (2006) Improved antioxidant activity of superoxide dismutase by direct chemical modification. J Control Release 111:204–211
108. Schumann J, Wolf D, Pahl A, Brune K, Papadopoulos T, van Rooijen N, Tiegs G (2000) Importance of Kupffer cells for T-cell-dependent liver injury in mice. Am J Pathol 157:1671–1683
109. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H (2000) The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+T cells in T helper 1 immune responses. Immunol Rev 174:35–46
110. Kelly C, Jefferies C, Cryan SA (2011) Targeted liposomal drug delivery to monocytes and macrophages. J Drug Deliv. http://dx.doi.org/10.1155/2011/727241
111. Dong L, Gao S, Diao H, Chen J, Zhang J (2007) Galactosylated low molecular weight chitosan as acarrier delivering oligonucleotides to Kupffer cells instead of hepatocytes in vivo. J Biomed Mater Res A 84(3):777–784
112. Miyoshi E, Moriwaki K, Terao N, Tan CC, Terao M, Nakagawa T, Matsumoto H, Shinzaki S, Kamada Y (2012) Fucosylation is a promising target for cancer diagnosis and therapy. Biomolecules 2:34
113. Hagiwara SI, Takeya M, Suzuki H, Kodama T, van der Laan LJW, Kraal G, Kitamura N, Takahashi K (1999) Role of macrophage scavenger receptors in hepatic granuloma formation in mice. Am J Pathol 154(3):705–720
114. Van Rooyen DM, Larter CZ, Haigh WG et al (2011) Hepatic free cholesterolaccumulates in obese, diabetic mice and causes nonalcoholicsteatohepatitis. Gastroenterology 141: 1393–1403
115. Ramprasad MP, Fischer W, Witztum JL, Sambrano GR, Quehenberger O, Steinberg D (1995) The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68. Proc Natl Acad Sci U S A 92(21):9580–9584
116. Rensen PCN, Gras JCE, Lindfors EK, van DijkKW JJW, van BerkelTJC BEAL (2006) Selective targeting of liposomes to macrophages using a ligand with high affinity for the macrophage scavenger receptor class A. Curr Drug Discov Technol 3:135–144
117. Kamps JAAM, Scherphof GL (1997) Massive targeting of liposomes, surface-modified with anionized albumins, to hepatic endothelial cells. Proc Natl Acad Sci U S A 94:11681–11685
118. Terpstra V, van Berkel TJ (2000) Scavenger receptors on liver Kupffer cells mediate the in vivo uptake of oxidatively damaged red blood cells in mice. Blood 95(6):2157–2163
119. Lovdal T, Andersen E, Brech A, Berg T (2000) Fc receptor mediated endocytosis of small soluble immunoglobulin G immune complexes in Kupffer and endothelial cells from rat liver. J Cell Sci 113:3255–3266
120. Peterson TC (1992) Mode of communication between Kupffer cells and Hepatocytes under normal and pathological conditions. In: Billiar TR, Curran RD (eds) Hepatocyte and Kupffer cell interactions. CRC, Boca Raton
121. Duryee MJ, Freeman TL, Willis MS, Hunter CD, Hamilton BC, Suzuki H, Tuma DJ, Klassen LW, Thiele GM (2005) Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins. Mol Pharmacol 68:1423–1430
122. Schledzewski K, Geraud C, Arnold B, Wang S, Gröne HJ, Kempf T, Wollert KC, Straub BK, Schirmacher P, Demory A, Schönhaber H, Gratchev A, Dietz L, Thierse HJ, Kzhyshkowska J, Goerdt S (2011) Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors. J Clin Invest 121(2):703–714

123. Friedman SL, Bansal MB (2006) Reversal of hepatic fibrosis—fact or fantasy? Hepatology 43:S82–S88
124. Fischer HD, Gonzalez-Noriega A, Sly WS, Morré DJ (1980) Phosphomannosyl-enzyme receptors in rat liver. Subcellular distribution and role in intracellular transport of lysosomal enzymes. J Biol Chem 255(20):9608–9615
125. Ghosh P, Dahms NM, Kornfeld S (2003) Mannose 6-phosphate receptors: new twists in the tale. Nat Rev Mol Cell Biol 4(3):202–212
126. Beljaars L, Olinga P, Molema G, de Bleser P, Geerts A, Groothuis GM, Meijer DK, Poelstra K (2001) Characteristics of the hepatic stellate cell-selective carrier mannose6-phosphate modified albumin (M6P28-HSA). Liver 21:320–328
127. Adrian JE, Kamps JA, Poelstra K, Scherphof GL, Meijer DK, Kaneda Y (2007) Delivery of viral vectors to hepatic stellate cells in fibrotic livers using HVJ envelopes fused with targeted liposomes. J Drug Target 15:75–82
128. Lepreux S, Bioulac-Sage P, Gabbiani G, Sapin V, Housset C, Rosenbaum J, Balabaud C, Desmoulière A (2004) Cellular retinol-binding protein-1 expression in normal and fibrotic/cirrhotic human liver: different patterns of expression in hepatic stellate cells and (myo) fibroblast subpopulations. J Hepatol 40(5):774–780
129. Sato Y, Murase K, Kato J, Kobune M, Sato T, Kawano Y, Takimoto R, Takada K, Miyanishi K, Matsunaga T, Takayama T, Niitsu Y (2008) Resolution ofliver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against acollagen-specific chaperone. Nat Biotechnol 26:431–442
130. Beljaars L, Molema G, Schuppan D, Geerts A, De Bleser PJ, Weert B, Meijer DK, Poelstra K (2000) Successful targeting to rat hepatic stellate cells using albumin modified with cyclic peptides that recognize the collagen type VI receptor. J Biol Chem 275:12743–12751
131. Du SL, Pan H, Lu WY, Wang J, Wu J, Wang JY (2007) Cyclic Arg-Gly-Asp peptide labeled liposomes for targeting drug therapy of hepatic fibrosis in rats. J Pharmacol Exp Ther 322:560–568
132. Wieckowska A, McCullough AJ, Feldstein AE (2007) Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. Hepatology 46:582–589
133. Wisse E et al (2008) The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. Gene Ther 15:1193–1199
134. Sorensen AL, Rumjantseva V, Nayeb-Hashemi S, Clausen H, Hartwig JH, Wandall HH, Hoffmeister KM (2009) Role of sialic acid for platelet life span: exposure of β-galactose results in the rapid clearance of platelets from the circulation by Asialoglycoprotein receptor—expressing liver macrophages and hepatocytes. Blood 114:1645–1654
135. Baenziger JU, Maynard Y (1980) Human hepatic lectin. Physiochemical properties and specificity. J Biol Chem 255:4607–4613
136. Iobst ST, Drickamer K (1996) Selective sugar binding to the carbohydrate recognition domains of the rat hepatic and macrophage asialoglycoprotein receptors. J Biol Chem 271(12):6686–6693
137. Fuhrer C, Geffen I, Huggel K, Spiess M (1994) The two subunits of the asialoglycoprotein receptor contain different sorting information. J Biol Chem 269(5):3277–3282
138. Ashwell G, Harford J (1982) Carbohydrate-specific receptors of the liver. Annu Rev Biochem 51:531–554
139. Murao A, Nishikawa M, Managit C, Wong J, Kawakami S, Yamastuta F, Hashida M (2002) Targeting efficiency of galactosylated liposomes to hepatocytes in vivo: effect of lipid composition. Pharm Res 19:1808–1813
140. Managit C, Kawakami S, Nishikawa M, Yamashita F, Hashida M (2003) Targeted and sustained drug delivery using PEGylated galactosylated liposomes. Int J Pharm 266(1–2):77–84
141. Managit C, Kawakami S, Yamashita F, Hashida M (2005) Effect of galacatose density on asialoglycoprotein receptor mediated uptake of galactosylated liposomes. J Pharm Sci 94(10):2266–2275
142. Bijsterbosch MK, Bernini F, Bakkeren HF, Gotto AM, Smith LC, Van Berkel TJ (1991) Enhanced hepatic uptake and processing of cholesterol esters from low density lipoprotein by specific lactosaminated fab fragments. Arterioscler Thromb Vasc Biol 11:1806–1813

143. Sliedregt LAJM, Rensen PCN, Rump ET, van Santbrink PJ, Bijsterbosch MK, Valentijn ARPM, van der Marel GA, van Boom JH, van Berkel TJC, Biessen EAL (1999) Design and synthesis of novel amphiphilic dendritic galactosides for selective targeting of liposomes to the hepatic asialoglycoprotein receptor. J Med Chem 42:609–618
144. Zhou X, Zhang M, Yung B, Li H, Zhou C, Lee LJ, Lee RJ (2012) Lactosylated liposomes for targeted delivery of doxorubicin to hepatocellular carcinoma. Int J Nanomedicine 7:5465–5547
145. Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S, Kerr DJ (2002) Cancer research campaign phase I/II clinical trials committee. Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin. J Clin Oncol 20(6):1668–1676
146. D'Souza AA, Jain P, Galdhar CN, Samad A, Degani MS, Devarajan PV (2013) Comparative in silico-in vivo evaluation of ASGP-R ligands for hepatic targeting of curcumin gantrez nanoparticles. AAPS J 15(3):696–706
147. Popielarski SR, Hu-Lieskovan S, French SW, Triche TJ, Davis ME (2005) A nanoparticle-based model delivery system to guidethe rational design of gene delivery to the liver. 2. In vitro and invivo uptake results. Bioconjug Chem 16(5):1071–1080
148. Wu F, Wuensch SA, Azadniv M, Ebrahimkhani MR, Crispe IN (2009) Galactosylated LDL nanoparticles: a novel targetingdelivery system to deliver antigen to macrophages and enhance antigen specific T cell responses. Mol Pharm 6(5):1506–1517
149. Biessen EAL, Bakkeren HF, Beuting DM, Kuiper J, Van Berkel TJC (1994) Ligand size is a major determinant of high-affinity binding of fucose- and galactose-exposing (lipo)proteins by the hepatic fucose receptor. Biochem J 299:291–296
150. Schlepper-Schafer J, Hulsmann D, Djovkar A, Meyer HE, Herbertz L, Kolb H, Kolb-Bachofen V (1986) Endocytosis via galactose receptors in vivo. Ligand size directs uptake by hepatocytes and/or liver macrophages. Exp Cell Res 165:494–506
151. Nishikawa M, Takemura S, Takakura Y, Hashida M (1998) Targeted delivery of plasmid DNA to hepatocytes in vivo: optimization of the pharmacokinetics of plasmid dna/galactosylated poly(L-Lysine) complexes by controlling their physicochemical properties. J Pharmacol Exp Ther 287:408–415
152. Negishi M, Irie A, Nagata N, Ichikawa A (1991) Specific binding of glycyrrhetinic acid to the rat liver membrane. Biochim Biophys Acta 1066:77–82
153. Lin AH, Liu Y, Huang Y, Sun J, Wu Z, Zhang X, Ping Q (2008) Glycyrrhizin surface modified chitosan nanoparticles for hepatocyte-targeted delivery. Int J Pharm 359:247–253
154. Akao T (2000) Differences in the metabolism of glycyrrhizin, glycyrrhetic acid and glycyrrhetic acid monoglucuronide by human intestinal flora. Biol Pharm Bull 23(12): 1418–1423
155. Huang W, Wang W, Wang P, Zhang CN, Tian Q, Zhang Y, Wang XH, Cha RT, Wang CH, Yuan Z (2011) Glycyrrhetinic acid-functionalized degradable micelles as liver-targeted drug carrier. J Mater Sci Mater Med 22(4):853–863
156. Shi L, Tang C, Yin C (2012) Glycyrrhizin-modified O-carboxymethyl chitosan nanoparticles as drug vehicles targeting hepatocellular carcinoma. Biomaterials 33(30):7594–7604
157. Tian Q, Wang X, Wang W, Zhang C, Liu Y, Yuan Z (2010) Insight into glycyrrhetinic acid: the role of the hydroxyl group on liver targeting. Int J Pharm 400:153–157
158. Tian Q, Wang X, Wang W, Zhang C, Yuan Z, Chen X (2011) Understanding the role of the C3-hydroxyl group in glycyrrhetinic acid on liver targeting. J Control Release 152:e192–e269
159. Tian Q, Wang XH, Wang W, Zhang CN, Wang P, Yuan Z (2012) Self-assembly and liver targeting of sulfated chitosan nanoparticles functionalized with glycyrrhetinic acid. Nanomedicine 8:870–879
160. Tian Q, Zhang CN, Wang XH, Wang W, Huang W, Wang CH, Yuan Z, Liu M, Wan HY, Tang H, Cha RT (2010) Glycyrrhetinic acid-modified chitosan/poly(ethylene glycol) nanoparticles for liver-targeted delivery. Biomaterials 31:4748–4756
161. Wu F, Xu T, Liu C, Chen C, Song X, Zheng Y, He G (2013) Glycyrrhetinic acid-poly(ethylene glycol)-glycyrrhetinic acid tri-block conjugates based self-assembled micelles for hepatic targeted delivery of poorly water soluble drug. Scientific World Journal 2013:913654

162. Mao SJ, Hou SX, He R, Zhang LK, Wei DP, Bi YQ, Jin H (2005) Uptake of albumin nanoparticle surface modified with glycyrrhizin by primary cultured rat hepatocytes. World J Gastroenterol 11(20):3075–3079
163. Zhang L, Yao J, Zhou J, Wang T, Zhang Q (2013) Glycyrrhetinic acid-graft-hyaluronic acid conjugate as a carrier for synergistic targeted delivery of antitumor drugs. Int J Pharm 441(1–2):654–664
164. Li FQ, Su H, Chen X, Qin XJ, Liu JY, Zhu QG, Hu JH (2009) Mannose 6-phosphate-modified bovine serum albumin nanoparticles for controlled and targeted delivery of sodium ferulate for treatment of hepatic fibrosis. J Pharm Pharmacol 61:1155–1161
165. Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L (2002) Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum Gene Ther 13:243–260
166. Haisma HJ, Bellu AR (2011) Pharmacological interventions for improving adenovirus usage in gene therapy. Mol Pharm 8:50–55
167. Kaneda Y (2001) Improvements in gene therapy technologies. Mol Urol 5:85–89
168. Kawashita Y, Fujioka H, Ohtsuru A, Kaneda Y, Kamohara Y, Kawazoe Y, Yamashita S, Kanematsu T (2005) The efficacy and safety of gene transfer into the porcine liver in vivo by HVJ (Sendai virus) liposome. Transplantation 80:1623–1629
169. Yamada T, Iwasaki Y, Tada H, Iwabuki H, Chuah MKL, VandenDriessche T, Fukuda H, Kondo A, Ueda M, Seno M, Tanizawa K, Kuroda S (2003) Nanoparticles for the delivery of genes and drugs to human hepatocytes. Nat Biotechnol 21:885–890
170. Smith T, Idamakanti N, Kylefjord H, Rollence M, King L, Kaloss M, Kaleko M, Stevenson SC (2002) In vivo hepatic adenoviral gene delivery occurs independently of the coxsackie virus-adenovirus receptor. Mol Ther 5(6):770–779
171. Xu Z, Tian J, Smith JS, Byrnes AP (2008) Clearance of adenovirus by kupffer cells is mediated by scavenger receptors, natural antibodies, and complement. J Virol 82(23):11705–11713
172. Gullberg D, Turner DC, Borg TK, Terracio L, Rubin K (1990) Different beta 1-integrin collagen receptors on rat hepatocytes and cardiac fibroblasts. Exp Cell Res 190(2): 254–264
173. Chen K, Chen X (2011) Integrin targeted delivery of chemotherapeutics. Theranostics 1:189–200
174. Marelli UK, Rechenmacher F, Sobahi TR, Mas-Moruno C, Kessler H (2013) Tumor targeting via integrin ligands. Front Oncol 3:222
175. Murphy EA, Majeti BK, Barnes LA, Makale M, Weis SM, Lutu-Fuga K, Wrasidlo W, Cheresh DA (2008) Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. Proc Natl Acad Sci U S A 105(27):9343–9348
176. Brown MS, Goldstein JL (1976) Receptor-mediated control of cholesterol metabolism. Science 191:150–154
177. Pieper-Furst U, Lammert F (2013) Low-density lipoprotein receptors in liver: old acquaintances and a newcomer. Biochim Biophys Acta 1831(7):1191–1198
178. Ye Q, Chen Y, Lei H, Liu Q, Moorhead JF, Varghese Z, Ruan XZ (2009) Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation. Inflamm Res 58:809–818
179. Kamps JA, Kruijt JK, Kuiper J, Van Berkel TJ (1991) Uptake and degradation of human low-density lipoprotein by human liver parenchymal and Kupffer cells in culture. Biochem J 276(1):135–140
180. Rui M, Guo W, Ding Q, Wei X, Xu J, Xu Y (2012) Recombinant high-density lipoprotein nanoparticles containing gadolinium-labeled cholesterol for morphologic and functional magnetic resonance imaging of the liver. Int J Nanomedicine 7:3751–3768
181. Jin H, Lovell JF, Chen J, Lin Q, Ding L, Ng KK, Pandey RK, Manoharan M, Zhang Z, Zheng G (2012) Mechanistic insights into LDL nanoparticle-mediated siRNA delivery. Bioconjug Chem 23(1):33–41
182. Wan C, Allen TM, Cullis PR (2013) Lipid nanoparticle delivery systems for siRNA-based therapeutics. Drug Deliv Transl Res 4:74–83

183. Kim SI, Shin D, Choi TH, Lee JC, Cheon GJ, Kim KY, Park M, Kim M (2007) Systemic and specific delivery of small interfering RNSs to the liver mediated by apolipoprotein A-I. Mol Ther 15:1145–1152
184. Kim SI, Shin D, Lee H, Ahn BY, Yoon Y, Kim M (2009) Targeted delivery of siRNA against hepatitis C virus by apolipoprotein A-I-bound cationic liposomes. J Hepatol 50:479–488
185. Volpes R, Van den Oord JJ, Desmet VJ (1990) Adhesive molecules in liver disease: immune histochemical distribution of thrombospondin receptors in chronic HBV infection. J Hepatol 10:297–304
186. Feng M, Cai Q, Huang H, Zhou P (2008) Liver targeting and anti-HBV activity of reconstituted HDL-acyclovir palmitate complex. Eur J Pharm Biopharm 68:688–693
187. Reddy LH, Couvreur P (2011) Nanotechnology for therapy and imaging of liver diseases. J Hepatol 55(6):1461–1466
188. Suzuki M, Fujimoto Y, Suzuki Y, Hosoki Y, Saito H, Nakayama K, Ohtake T, Kohgo Y (2004) Induction of transferrin receptor by ethanol in rat primary hepatocyte culture. Alcohol Clin Exp Res 28:98S–105S
189. Suzuki Y, Saito H, Suzuki M, Hosoki Y, Sakurai S, Fujimoto Y, Kohgo Y (2002) Up-regulation of transferrin receptor expression in hepatocytes by habitual alcohol drinking is implicated in hepatic iron overload in alcoholic liver disease. Alcohol Clin Exp Res 26:26S–31S
190. Qian ZM, Li H, Sun H, Ho K (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54(4):561–587
191. He Q, Yuan WM, Liu J, Zhang ZR (2008) Study on in vivo distribution of liver-targeting nanoparticles encapsulating thymidine kinase gene (TK gene) in mice. J Mater Sci Mater Med 19:559–565
192. Kramer W, Wess G, Schubert G, Bickel M, Girbig F, Gutjahr U, Kowalewski S, Baringhaus KH, Enhsen A, Glombik H, Mullner S, Neckermann G, Schulz S, Petzinger E (1992) Liver-specific drug targeting by coupling to bile acids. J Biol Chem 267(26):18598–18604
193. Vaquero J, Briz O, Herraez E, Muntané J, Marin JJ (2013) Activation of the nuclear receptor FXR enhances hepatocyte chemoprotection and liver tumor chemoresistance against genotoxic compounds. Biochim Biophys Acta 1833(10):2212–2219
194. Annoni A, Goudy K, Akbarpour M, Naldini L, Roncarolo MG (2013) Immune responses in liver-directed lentiviral gene therapy. Transl Res 161:230–240
195. Mallat A, Teixeira-Clerc F, Lotersztajn S (2013) Cannabinoid signaling and liver therapeutics. J Hepatol 59(4):891–896
196. He ZG, Cai HJ, Chen XY, Wang N (1990) Modulation of rat Kupffer cells on high density lipoprotein receptors on hepatocytes. Sci China B 33(5):584–591
197. Jindadamrongwech S, Thepparit C, Smith DR (2004) Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 149(5):915–927
198. Thepparit C, Smith DR (2004) Serotype-specific entry of dengue virus into liver cells: Identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. J Virol 78(22):12647–12656
199. Cabrera-Hernandez A, Smith DR (2005) Mammalian dengue virus receptors. Dengue Bull 29(662):119–135
200. Hidari KI, Suzuki T (2011) Dengue virus receptor. Trop Med Health 39(4):37–43
201. Ding X, Saxena NK, Lin S, Gupta NA, Anania FA (2006) Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. Hepatology 43(1):173–181
202. Gupta NA, Mells J, Dunham RM, Grakoui A, Handy J, Saxena NK, Anania FA (2010) Glucagon-like peptide-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro by modulating elements of the insulin signaling pathway. Hepatology 51(5):1584–1592
203. Kim JS, Lemasters JJ (2006) Opioid receptor-independent protection of ischemic rat hepatocytes by morphine. Biochem Biophys Res Commun 351(4):958–964
204. Pacher P, Batkai S, Kunos G (2006) The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 58(3):389–462

205. Pertwee RG (2006) The pharmacology of cannabinoid receptors and their ligands: an overview. Int J Obes (Lond) 30:S13–S18
206. Donnenberg VS, Donnenberg AD (2005) Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. J Clin Pharmacol 45:872–877
207. Breuhahn K, Longerich T, Schirmacher P (2006) Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene 25:3787–3800
208. Merle P, de la Monte S, Kim M, Herrmann M, Tanaka S, Von Dem Bussche A, Kew MC, Trepo C, Wands JR (2004) Functional consequences of frizzled-7 receptor overexpression in human hepatocellular carcinoma. Gastroenterology 127:1110–1122
209. Marquardt JU, Galle PR, Teufel A (2012) Molecular diagnosis and therapy of hepatocellular carcinoma (HCC): an emerging field for advanced technologies. J Hepatol 56:267–275
210. Oishi N, Wang XW (2011) Novel therapeutic strategies for targeting liver cancer stem cells. Int J Biol Sci 7(5):517–535
211. Seo SB, Yang J, Hyung W, Cho EJ, Lee TI, Song YJ, Yoon HG, Suh JS, Huh YM, Haam S (2007) Novel multifunctional PHDCA/PEI nano-drug carriers for simultaneous magnetically targeted cancer therapy and diagnosis via magnetic resonance imaging. Nanotechnology 18:1–8
212. Marin A, Sun H, Husseini GA, Pitt WG, Christensen DA, Rapoport NY (2000) Drug delivery in pluronic micelles: effect of high-frequency ultrasound on drug release from micelles and intracellular uptake. J Control Release 84:39–47
213. Bawa P, Pillay V, Choonara YE, du Toit LC (2009) Stimuli-responsive polymers and their applications in drug delivery. Biomed Mater 4(2):022001
214. Qing G, Li M, Deng L, Lv Z, Ding P, Sun T (2013) Smart drug release systems based on stimuli-responsive polymers. Mini Rev Med Chem 13(9):1369–1380
215. Lee ES, Kun N, Bae YH (2005) Super pH-Sensitive multifunctional polymeric micelle. Nano Lett 5:325–329
216. Chen Q, Tong S, Dewhirst MW, Yuan F (2004) Targeting tumor microvessels using doxorubicin encapsulated in a novel thermosensitive liposome. Mol Cancer Ther 3:1311–1317
217. Kono K (2001) Thermosensitive polymer-modified liposomes. Adv Drug Deliv Rev 53:307–319
218. Johnson RP, Jeong YI, John JV, Chung CW, Kang DH, Selvaraj M, Suh H, Kim I (2013) Dual stimuli-responsive poly(N-isopropylacrylamide)-b-poly(L-histidine) chimeric materials for the controlled delivery of doxorubicin into liver carcinoma. Biomacromolecules 14(5):1434–1443
219. Ponce AM, Vujaskovic Z, Yuan F, Needham D, Dewhirst MW (2006) Int J Hyperthermia 22:205
220. Khandare JJ, Minko T (2006) Antibodies and peptides in cancer therapy. Crit Rev Ther Drug Carrier Syst 25:401–435
221. Mastrobattista E, Koning GA, Storm G (1999) Immunoliposomes for the targeted delivery of antitumor drugs. Adv Drug Deliv Rev 40:103–127
222. Patil RR, Guhagarkar SA, Devarajan PV (2008) Engineered nanocarriers of doxorubicin: a current update. Crit Rev Ther Drug Carrier Syst 25(1):1–61
223. Trahtenherts A, Benhar I (2009) An internalizing antibody specific for the human asialoglycoprotein receptor. Hybridoma (Larchmt) 28(4):225–233
224. Coulstock E, Sosabowski J, Ovečka M, Prince R, Goodall L, Mudd C, Sepp A, Davies M, Foster J, Burnet J, Dunlevy G, Walker A (2013) Liver-targeting of interferon-alpha with tissue-specific domain antibodies. PLoS One 8(2):e57263
225. Feng M, Ho M (2014) Glypican-3 antibodies: a new therapeutic target for liver cancer. FEBS Lett 588(2):377–382
226. Jin C, Bai L, Li H, He Y, An J, Dou K (2013) Paclitaxel-loaded nanoparticles decorated with anti-CD133 antibody for targeting liver cancer stem cells. J Control Release 172(1):e20–e21
227. Douglass A, Wallace K, Koruth M, Barelle C, Porter AJ, Wright MC (2010) Using a recombinant single chain antibody for targeting liver myofibroblasts with anti-fibrogenic therapeutics. Arab J Gastroenterol 10:S3–S6

228. Elrick LJ, Leel V, Blaylock MG, Duncan L, Drever MR, Strachan G, Charlton KA, Koruth M, Porter AJ, Wright MC (2005) Generation of a monoclonal human single chain antibody fragment to hepatic stellate cells—a potential mechanism for targeting liver anti-fibrotic therapeutics. J Hepatol 42:888–896
229. Praetorius NP, Mandal TK (2007) Engineered nanoparticles in cancer therapy. Recent Pat Drug Deliv Formul 1(1):37–51
230. Mishra N, Yadav NP, Rai VK, Sinha P, Yadav KS, Jain S, Arora S (2013) Efficient hepatic delivery of drugs: novel strategies and their significance. Biomed Res Int 2013:382184
231. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC (2008) Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther 83(5):761
232. Hirabayashi H, Nishikawa M, Takakura Y, Hashida M (1996) Development and pharmacokinetics of galactosylated poly-L-glutamic acid as a biodegradable carrier for liver-specific drug delivery. Pharm Res 13(6):880–884
233. Palumbo E (2009) PEG-interferon in acute and chronic hepatitis C: a review. Am J Ther 16(6):573–578
234. Arangoa MA, Duzgunes N, de Ilarduya CT (2003) Increased receptor-mediated gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes. Gene Ther 10:5–14
235. Dirk L, Edith J, Andreas Z (2004) Drug delivery of oligonucleotides by peptides. Eur J Pharm Biopharm 58(2):237–251
236. Gu W, Wu C, Chen J, Xiao Y (2013) Nanotechnology in the targeted drug delivery for bone diseases and bone regeneration. Int J Nanomedicine 8(1):2305–2317
237. Allen TM, Cullis PR (2013) Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev 65:36–48
238. Bae YH, Park K (2011) Targeted drug delivery to tumors: myths, reality and possibility. J Control Release 153(3):198–205
239. Wei H, Huang J, Yang J, Zhang X, Lin L, Xue E, Chen Z (2013) Ultrasound exposure improves the targeted therapy effects of galactosylated docetaxel nanoparticles on hepatocellular carcinoma xenografts. PLoS One 8(3):e58133
240. Czaja AJ (2013) Hepatocellular carcinoma and other malignancies in autoimmune hepatitis. Dig Dis Sci 58(6):1459–1476
241. Wang H, Chen L (2013) Tumor microenviroment and hepatocellularcarcinoma metastasis. J Gastroenterol Hepatol 28(S1):43–48
242. Edens HA, Levi BP, Jaye DL, Walsh S, Reaves TA, Turner JR, Nusrat A, Parkos CA (2002) Neutrophil transepithelial migration: evidence for sequential, contact-dependent signaling events and enhanced paracellular permeability independent of transjunctional migration. J Immunol 169:476–486
243. Iyer AK, Khaled G, Fang J, Maeda H (2006) Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discov Today 11(17–18):812–818
244. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 65:271
245. Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, Jain RK (1995) Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res 55:3752
246. Moghimi SM, Hunter AC, Murray JC (2001) Long-circulating and target-specific nanoparticles: theory to practice. Pharmacol Rev 53(2):283–318
247. Cho K, Wang X, Nie S, Chen Z, Shin DM (2008) Therapeutic nanoparticles for drug delivery in cancer. Clin Cancer Res 14:1310
248. Villa R, Cerroni B, Viganòa L, Margheritelli S, Abolafio G, Oddo L, Paradossi G, Zaffaroni N (2013) Targeted doxorubicin delivery by chitosan-galactosylated modified polymer microbubbles to hepatocarcinoma cells. Colloids Surf B Biointerfaces 110:434–442
249. Varshosaz J, Hassanzadeh F, Sadeghi H, Khan ZG, Rostami M (2013) Retinoic Acid Decorated albumin-chitosan nanoparticles for targeted delivery of doxorubicin hydrochloride in hepatocellular carcinoma. J Nanomaterials Article ID 254127, 12 p. http://dx.doi.org/10.1155/2013/254127

250. Zhang C, Wang W, Liu T, Wu Y, Guo H, Wang P, Wang Y, Yuan Z, Tian Q (2012) Doxorubicin loaded glycyrrhetinic acid-modified alginate nanoparticles for liver tumor chemotherapy. Biomaterials 33:2187–2196
251. Jain A, Jain K, Mehra NK, Jain NK (2013) Lipoproteins tethered dendrimeric nanoconstructs for effective targeting to cancer cells. J Nanopart Res 15:2003
252. Zhu XL, Du YZ, Yu RS, Liu P, Shi D, Chen Y, Wang Y, Huang FF (2013) Galactosylated chitosan oligosaccharide nanoparticles for hepatocellular carcinoma cell-targeted delivery of adenosine triphosphate. Int J Mol Sci 14(8):15755–15766
253. Greish K (2012) Enhanced permeability and retention effect for selective targeting of anti-cancer nanomedicine: are we there yet? Drug Discov Today Technol 9(2):e161–e166
254. Konno T, Maeda H, Iwai K, Maki S, Tashiro S, Uchida M, Miyauchi Y (1984) Selective targeting of anti-cancer drug and simultaneous image enhancement in solid tumors by arterially administered lipid contrast medium. Cancer 54:2367–2374
255. Maeda H (2001) SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. Adv Drug Deliv Rev 46(1–3):169–185
256. Yamaoka T, Tabata Y, Ikada Y (1994) Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. J Pharm Sci 83(4):601–606
257. Ruoslahti E, Bhatia SN, Sailor MJ (2010) Targeting of drugs and nanoparticles to tumors. J Cell Biol 188(6):759–768
258. Angulo-Barturen I, Santiago Ferrer S (2013) Humanised models of infection in the evaluation of anti-malarial drugs. Drug Discov Today Technol 10(3):e351–e357
259. Pradel G, Garapaty S, Frevert U (2004) Kupffer and stellate cell proteoglycans mediate malaria sporozoitetargeting to the liver. Comp Hepatol 3(Suppl 1):S47
260. Arica B, Ozer AY, Ercan MT, Hincal AA (1995) Characterization, in vitro and in vivo studies on primaquine diphosphate liposomes. J Microencapsul 12:469–485
261. Pirson P, Steiger RF, Trouet A, Gillet J, Herman F (1980) Primaquine liposomes in the chemotherapy of experimental murine malaria. Ann Trop Med Parasitol 74:383–391
262. Labhasetwar VD, Dorle AK (1990) Nanoparticles—a colloidal drug delivery system for primaquine and metronidazole. J Control Release 12:113–119
263. Dierling AM, Cui Z (2005) Targeting primaquine into liver using chylomicron emulsions for potential vivax malaria therapy. Int J Pharm 33:148–152
264. Bhadra D, Yadav AK, Bhadra S, Jain NK (2005) Glycodendrimeric nanoparticulate carriers of primaquine phosphate for liver targeting. Int J Pharm 295:221–233
265. Dolina JS, Sung SSJ, Novobrantseva TI, Nguyen TM, Hahn YS (2013) Lipidoid nanoparticles containing PD-L1 siRNA delivered in vivo enter Kupffer cells and enhance NK and CD8+ T Cell-mediated hepatic antiviral immunity. Mol Ther Nucleic Acids 2:e72
266. Midoux P, Pichon C, Yaouanc JJ, Jaffres PA (2009) Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. Br J Pharmacol 157(2):166–178
267. Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, Bertin SL, Reppen TW, Chu Q, Blokhin AV, Hagstrom JE, Wolff JA (2007) Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes. Proc Natl Acad Sci U S A 104:12982–12987
268. Craparo EF, Triolo D, Pitarresi G, Giammona G, Cavallaro G (2013) Galactosylated micelles for a ribavirin prodrug targeting to hepatocytes. Biomacromolecules 14(6):1838–1849
269. Lim DW, Yeom YI, Park TG (2000) Poly(DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. Bioconjug Chem 11:688–695
270. Szabo G, Bala S (2013) MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol 10:542–552
271. Giri N, Tomar P, Karwasara VS, Pandey RS, Dixit VK (2011) Targeted novel surface-modified nanoparticles for interferon delivery for the treatment of hepatitis B. Acta Biochim Biophys Sin 43:877–883
272. Rosen HR (2011) Chronic hepatitis C infection. N Engl J Med 364:2429–2438

273. Poelstra K, Prakash J, Beljaars L (2012) Drug targeting to the diseased liver. J Control Release 161:188–197
274. Jung J, Matsuzaki T, Tatematsu K, Okajima T, Tanizawa K, Kuroda S (2008) Bionanocapsule conjugated with liposomes for in vivo pinpoint delivery of various materials. J Control Release 126:255–264
275. Liu L, Hitchens TK, Ye Q, Wu Y, Barbe B, Prior DE, Li WF, Yeh FC, Foley LM, Bain DJ, Ho C (2013) Decreased reticuloendothelial system clearance and increased blood half-life and immune cell labeling for nano and micron sized superparamagnetic iron-oxide particles upon pre-treatment with Intralipid. Biochim Biophys Acta 1830(6):3447–3453
276. Liu YJ, Chen ZJ, Zhang N (2011) Novel nanovectors as liver targeting MRI contrast agents. J Chin Pharmaceut Sci 20:105–117
277. Yasuharu O, Ishida H, Hayashi A, Kamagata S, Hirobe S, Ishii K (2002) The mean transit time and functional imagein asialoglycoprotein receptor scintigraphy: a novel modality for evaluating theregional dynamic function of hepatocytes. J Nucl Med 43:1611–1615
278. Ni HM, Williams JA, Yang H, Shi YH, Fan J, Ding WX (2012) Targeting autophagy for the treatment of liver diseases. Pharmacol Res 66:463–474
279. Stern ST, Adiseshaiah PP, Crist RM (2012) Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part Fibre Toxicol 9:20
280. Sano A, Taylor ME, Leaning MS, Summerfield JA (1990) Uptake and processing of glycoproteins by isolated rat hepatic endothelial and Kupffer cells. J Hepatol 10(2):211–216
281. Opanasopit P, Nishikawa M, Yamashita F, Takakura Y, Hashida M (2001) Pharmacokinetic analysis of lectin-dependent biodistribution of fucosylated bovine serum albumin: a possible carrier for Kupffer cells. J Drug Target 9(5):341–351
282. Ahmed SS, Muro H, Nishimura M, Kosugi I, Tsutsi Y, Shirasawa H (1995) Fc receptors in liver sinusoidal endothelial cells in NZB/WF1 lupus mice: a histological analysis using soluble immunoglobulin G-immune complexes and a monoclonal antibody (2.4G2). Hepatology 22(1):316–324
283. Huang Z, Hoffmann FW, Fay JD, Hashimoto AC, Chapagain ML, Kaufusi PH, Hoffmann PR (2012) Stimulation of unprimed macrophages with immune complexes triggers a low output of nitric oxide by calcium-dependent neuronal nitric-oxide synthase. J Biol Chem 287(7):4492–4502
284. Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J, van Berkel TJC (2000) Hepatic and extrahepatic scavenger receptors function in relation to disease. Arterioscler Thromb Vasc Biol 20:1860–1872
285. Van Oosten M, van de Bilt E, van Berkel TJ, Kuiper J (1998) New scavenger receptor-like receptors for the binding of lipopolysaccharide to liver endothelial and Kupffer cells. Infect Immun 66(11):5107–5112
286. Van Berkel TJ, De Rijke YB, Kruijt JK (1991) Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. J Biol Chem 266(4):2282–2289
287. Cardarelli PM, Blumenstock FA, McKeown-Longo PJ, Saba TM, Mazurkiewicz JE, Dias JA (1990) High-affinity binding of fibronectin to cultured Kupffer cells. J Leukoc Biol 48(5):426–437
288. Hansen B, Arteta B, Smedsrød B (2002) The physiological scavenger receptor function of hepatic sinusoidal endothelial and Kupffer cells is independent of scavenger receptor class A type I and II. Mol Cell Biochem 240(1–2):1–8
289. Li R, Oteiza A, Sorensen KK, McCourt P, Olsen R, Smedsrod B, Svistounov D (2011) Role of liver sinusoidal endothelial cells and stabilins in elimination of oxidized low-density lipoproteins. Am J Physiol Gastrointest Liver Physiol 300:G71–G81
290. Mousavi SA, Sporstol M, Fladeby C, Kjeken R, Barois N, Berg T (2007) Receptor-mediated endocytosis of immune complexes in rat liver sinusoidal endothelial cells is mediated by FcRIIb2. Hepatology 46(3):871–884
291. Muro H, Shirasawa H, Kosugi I, Nakamura S (1993) Defect of Fc receptors and phenotypical changes in sinusoidal endothelial cells in human liver cirrhosis. Am J Pathol 143(1):105–120

292. McGary CT, Raja R, Weigel PH (1989) Endocytosis of hyaluronic acid by rat liver endothelial cells.evidence for receptor recycling. Biochem J 257(3):875
293. Stefanovic L, Stefanovic B (2012) Role of cytokine receptor-like factor 1 in hepatic stellate cells and fibrosis. World J Hepatol 4(12):356–364
294. March S, Graupera M, Sarrias MR, Lozano F, Pizcueta P, Bosch J, Engel P (2007) Identification and functional characterization of the hepatic stellate cell CD38 cell surface molecule. Am J Pathol 170(1):176–187
295. Bridle KR, Crawford DH, Rammer GA (2003) Identification and characterization of the hepatic stellate cell transferrin receptor. Am J Pathol 162(5):1661–1667
296. Olaso E, Ikeda K, Eng FJ, Xu L, Wang LH, Lin HC, Friedman SL (2001) DDR2 receptor promotes MMP-2-mediated proliferation and invasion by hepatic stellate cells. J Clin Invest 108(9):1369–1378
297. Leyland H, Gentry J, Arthur MJ, Benyon RC (1996) The plasminogen-activating system in hepatic stellate cells. Hepatology 24(5):1172–1178
298. Zhang LP, Takahara T, Yata Y, Furui K, Jin B, Kawada N, Watanabe A (1999) Increased expression of plasminogen activator and plasminogen activator inhibitor during liver fibrogenesis of rats: role of stellate cells. J Hepatol 31(4):703–711
299. Bataller R, Nicolas JM, Ginès P, Esteve A, Nieves Görbig M, Garcia-Ramallo E, Pinzani M, Ros J, Jiménez W, Thomas AP, Arroyo V, Rodes J (1997) Arginine vasopressin induces contraction and stimulates growth of cultured human hepatic stellate cells. Gastroenterology 113(2):615–624
300. Hinglais N, Kazatchkine MD, Mandet C, Appay MD, Bariety J (1989) Human liver Kupffer cells express CR1, CR3, and CR4 complement receptor antigens. An immunohistochemical study. Lab Invest 61(5):509–514
301. Yan J, Vetvicka V, Xia Y, Hanikyrova M, Mayadas TN, Ross GD (2000) Critical role of Kupffer cell CR3 (CD11b/rCD18) in the clearanceof IgM-opsonized erythrocytes or soluble b-glucan. Immunopharmacology 46:39–54
302. Hirose S, Ise H, Uchiyama M, Cho CS, Akaike T (2001) Regulation of asialoglycoprotein receptor expression in the proliferative state of hepatocytes. Biochem Biophys Res Commun 287(3):675–681
303. Gumpricht E, Dahl R, Devereaux MW, Sokol RJ (2005) Licorice compounds glycyrrhizin and 18β-glycyrrhetinic acid are potent modulators of bile acid-induced cytotoxicity in rat hepatocytes. J Biol Chem 280:10556–10563
304. Shu Y, Xiao L, Zhao J, Zhu H, Zhou Z, Cheng N (1999) Change of high density lipoprotein receptor of hepatocyte during cholesterol gallstone formation in rabbit model. Hua Xi Yi Ke Da Xue Xue Bao 30(3):296–298
305. Albecka A, Belouzard S, Op de Beeck A, Descamps V, Goueslain L, Bertrand-Michel J, Terce F, Duverlie G, Rouillé Y, Dubuisson J (2012) Role of low-density lipoprotein receptor in the hepatitis C virus life cycle. Hepatology 55(4):998–1007
306. Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, Roitelman J, Barbaras R, Graber P, Ghersa P, Smolarsky M, Funaro A, Malavasi F, Larrey D, Coste J, Fabre JM, Sa-Cunha A, Maurel P (2007) The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J Hepatol 46(3):411–419
307. Nenseter MS, Myklebost O, Blomhoff R, Drevon CA, Nilsson A, Norum KR, Berg T (1989) Low-density-lipoprotein receptors in different rabbit liver cells. Biochem J 261(2):587–593
308. Barth H, Cerino R, Arcuri M, Hoffmann M, Schürmann P, Adah MI, Gissler B, Zhao X, Ghisetti V, Lavezzo B, Blum HE, von Weizsäcker F, Vitelli A, Scarselli E, Baumert TF (2005) Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. J Virol 79(9):5774–5785
309. Hoekstra M, Van Berkel TJ, Van Eck M (2010) Scavenger receptor BI: a multi-purpose player in cholesterol and steroid metabolism. World J Gastroenterol 16(47):5916–5924
310. Akiko HK, Hiroshi S, Nobu A (1984) Increase of transferrin receptors in hepatocytes during rat liver regeneration. Int J Biochem 16(6):601–605

311. Kishimoto T, Tavassoli M (1986) Recovery of transferrin receptors on hepatocytes membrane after collagenase perfusion. Biochem Biophys Res Commun 134:711–715
312. Rapisarda C, Puppi J, Hughes RD, Dhawan A, Farnaud S, Evans RW, Sharp PA (2010) Transferrin receptor 2 is crucial for iron sensing in human hepatocytes. Am J Physiol Gastrointest Liver Physiol 299:G778–G783
313. McClain DA, Olefsky JM (1988) Evidence for two independent pathways of insulin-receptor internalization in hepatocytes and hepatoma cells. Diabetes 37(6):806–815
314. Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol Cell 6(1):87–97
315. Emmett DS, Feranchak A, Kilic G, Puljak L, Miller B, Dolovcak S, McWilliams R, Doctor RB, Fitz JG (2008) Characterization of ionotrophic purinergic receptors in hepatocytes. Hepatology 47(2):698–705
316. Liu J, Zhou L, Xiong K, Godlewski G, Mukhopadhyay B, Tam J, Yin S, Gao P, Shan X, Pickel J, Bataller R, O'Hare J, Scherer T, Buettner C, Kunos G (2012) Hepatic cannabinoid receptor-1 mediates diet-induced insulin resistance via inhibition of insulin signaling and clearance in mice. Gastroenterology 142(5):1218–1228
317. Miyata R, Ueda M, Jinno H, Konno T, Ishihara K, Ando N, Kitagawa Y (2009) Selective targeting by preS1 domain of hepatitis B surface antigen conjugated with phosphorylcholine-based amphiphilic block copolymer micelles as a biocompatible, drug delivery carrier for treatment of human hepatocellular carcinoma with paclitaxel. Int J Cancer 124:2460
318. Li C, Zhang D, Guo H, Hao L, Zheng D, Shen J, Tian X, Zhang Q (2013) Preparation and characterization of galactosylated bovine serum albumin nanoparticles for liver-targeted delivery of oridonin. Int J Pharm 448:79–86
319. Bu L, Gan LC, Guo XQ, Chen FZ, Song Q, Gou XJ, Hou SX, Yao Q, Zhao Q (2013) Trans-resveratrol loaded chitosan nanoparticles modified with biotin and avidin to target hepatic carcinoma. Int J Pharm 452:355–362
320. Li F, Sun JY, Wang JY, Du SL, Lu WY, Liu M, Xie C, Shi JY (2008) Effect of hepatocyte growth factor encapsulated in targeted liposomes on liver cirrhosis. J Control Release 131:77–82
321. Li X, Wu Q, Chen Z, Gong X, Lin X (2008) Preparation, characterization and controlled release of liver-targeting nanoparticles from the amphiphilic random copolymer. Polymer 49:4769–4775
322. Hagens WI, Mattos A, Greupink R, de Jager-Krikken A, Reker-Smit C, van Loenen-Weemaes A, Gouw IA, Poelstra K, Beljaars L (2007) Targeting 15d-prostaglandin J2 to hepatic stellate cells: two options evaluated. Pharm Res 24:566
323. Mandal AK, Das S, BasuMK C, DasN RN (2007) Hepatoprotective activityof liposomal flavonoid against arsenite-induced liver fibrosis. J Pharmacol Exp Ther 320:994–1001
324. Takei Y, Maruyama A, Ferdous A, Nishimura Y, Kawano S, Ikejima K, Okumura S, Asayama S, Nogawa M, Hashimoto M, Makino Y, Kinoshita M, Watanabe S, Akaike T, Lemasters JJ, Sato N (2004) Targeted gene delivery to sinusoidal endothelial cells: DNA nanoassociate bearing hyaluronan-glycocalyx. FASEB J 18(6):699–701
325. Toriyabe N, Hayashi Y, Hyodo M, Harashima H (2011) Synthesis and evaluation of stearylated hyaluronic acid for the active delivery of liposomes to liver endothelial cells. Biol Pharm Bull 34:1084–1089
326. Kim EM, Jeong HJ, Park IK, Cho CS, Kim CG, Bom HS (2005) Hepatocyte-targeted nuclear imaging using 99mTc-galactosylated chitosan: conjugation, targeting, and biodistribution. J Nucl Med 46:141–145
327. Diebold SS, Plank C, Cotten M, Wagner E, Zenke M (2002) Mannose receptor-mediated gene delivery into antigen presenting dendritic cells. Somat Cell Mol Genet 27(1–6):65–74
328. Wang HX, Xiong MH, Wang YC, Zhu J, Wang J (2013) N-acetylgalactosamine functionalized mixed micellar nanoparticles for targeted delivery of siRNA to liver. J Control Release 166:106–114
329. Wang Y, Zhang X, Yu P, Li C (2013) Glycopolymer micelles with reducible ionic cores for hepatocytes-targeting delivery of DOX. Int J Pharm 441(1–2):170–180

330. Díez S, Navarro G, de ILarduya CT (2009) In vivo targeted gene delivery by cationic nanoparticles for treatment of hepatocellular carcinoma. J Gene Med 11(1):38–45
331. Singh KK, Vingkar SK (2008) Formulation, antimalarial activity and biodistribution of oral lipid nanoemulsion of primaquine. Int J Pharm 347(1–2):136–143
332. Vyas SP, Sihorkar V (2000) Endogenous carriers and ligands in nonimmunogenic site-specific drug delivery. Adv Drug Deliv Rev 43(2–3):101–164
333. Kim KS, Kim S, Beack S, Yang JA, Yun SH, Hahn SK (2012) In vivo real-time confocal microscopy for target-specific delivery of hyaluronic acid-quantum dot conjugates. Nanomedicine 8:1070–1073
334. Kikkeri R, Lepenies B, Adibekian A, Laurino P, Seeberger PH (2009) In vitro imaging and in vivo liver targeting with carbohydrate capped quantum dots. J Am Chem Soc 131:2110–2112
335. Park JO, Stephen Z, Sun C, Veiseh O, Kievit FM, Fang C, Leung M, Mok H, Zhang M (2011) Glypican-3 targeting of liver cancer cells using multifunctional nanoparticles. Mol Imaging 10(1):69–77
336. Kim EM, Jeong HJ, Kim SL, Sohn MH, Nah JW, Bom HS, Park IK, Cho CS (2006) Asialoglycoprotein-receptor-targeted hepatocyte imaging using 99mTc galactosylated chitosan. Nucl Med Biol 33:529–534
337. Ocampo-García BE, Ramírez Fde M, Ferro-Flores G, De León-Rodríguez LM, Santos-Cuevas CL, Morales-Avila E, de Murphy CA, Pedraza-López M, Medina LA, Camacho-López MA (2011) ^{99m}Tc-labelled gold nanoparticles capped with HYNIC-peptide/mannose for sentinel lymph node detection. Nucl Med Biol 38(1):1–11
338. Langereis S, de Lussanet QG, van Genderen MHP, Baces WH, Hackeng TM, van Engelshoven JM, Meijer EW (2004) Abstr Paper Am Chem Soc 228:U420–U420
339. Chen Z, Yu D, Wang S, Zhang N, Ma C, Lu Z (2009) Biocompatible nanocomplexes for molecular targeted MRI contrast agent. Nanoscale Res Lett 4:618–626
340. Yang SH, Heo D, Lee E, Kim E, Lim EK, Lee YH, Haam S, Suh JS, Huh YM, Yang J, Park SW (2013) Galactosylated manganese ferrite nanoparticles for targeted MR imaging of asialoglycoprotein receptor. Nanotechnology 24(47):475103
341. Vu-Quang H, Yoo MK, Jeong HJ, Lee HJ, Muthiah M, Rhee JH, Lee JH, Cho CS, Jeong YY, Park IK (2011) Targeted delivery of mannan-coated superparamagnetic iron oxide nanoparticles to antigen-presenting cells for magnetic resonance-based diagnosis of metastatic lymph nodes in vivo. Acta Biomater 7(11):3935–3945
342. Corbin IR, Li H, Chen J, Lund-Katz S, Zhou R, Glickson JD, Zheng G (2006) Low-density lipoprotein nanoparticles as magnetic resonance imaging contrast agents. Neoplasia 8(6):488–498
343. Barraud L, Merle P, Soma E, Lefrancois L, Guerret S, Chevallier M, Dubernet C, Couvreur P, Trépo C, Vitvitski L (2005) Increase of doxorubicin sensitivity by doxorubicin-loaded into nanoparticles for hepatocellular carcinoma cells in vitro and in vivo. J Hepatol 42:736–743
344. Fu S, Naing A, Moulder SL, Culotta KS, Madoff DC, Ng CS, Madden TL, Falchook GS, Hong DS, Kurzrock R (2011) Phase I trial of hepatic arterial infusion of nanoparticle albumin–bound paclitaxel: toxicity, pharmacokinetics, and activity. Mol Cancer Ther 10:1300
345. Wagner V, Dullaart A, Bock AK, Zweck A (2006) The emerging nanomedicine landscape. Nat Biotechnol 24:1211–1217
346. Apostolov EO, Shah SV, Ray D, Basnakian AG (2009) Scavenger receptors of endothelial cells mediate the uptake and cellular proatherogenic effects of carbamylated LDL. Arterioscler Thromb Vasc Biol 29(10):1622–1630
347. Chen ZJ, Yu DX, Liu CX, Yang XY, Zhang N, Ma CH, Song JB, Lu ZJ (2011) Gadolinium-conjugated PLA-PEG nanoparticles as liver targeted molecular MRI contrast agent. J Drug Target 19(8):657–665

Part IV
Cell- and Cell Organelle–Based Targeting

Chapter 7
Targeted Drug Delivery to the Mitochondria

Udita Agrawal, Rajeev Sharma, and Suresh P. Vyas

Abbreviations

CPPs	Cell penetrating peptides
CsA	Cyclosporin A
CTAB	Cetyltrimethylammonium bromide
DOPE	Di-oleoylphosphatidylethanolamine
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	Dioleoyl-1,2-diacyl-3-trimethylammoniumpropane
DOTMA	2,3-Bis-(oleoyl)oxipropyl-trimethyl ammonium chloride
GSH	Glutathione
Mce 6	Photosensitizer mesochlorine 6
MEND	Multifunctional envelope-type nano-device
MLS	Mitochondrial targeting sequences
MPPs	Mitochondria-penetrating peptides
mPTPCs	Mitochondrial permeability transition pore complexes
MTS	Mitochondrial targeting signal peptide
PAA	Polyacrylic acid
PAMAM	Poly(amidoamine) dendrimer
PLGA	Poly-lactide-co-glycolide
PT	Permeability transition
PTD	Protein transduction domain
PTPC	Permeability transition pore complex
SOD	Super-oxide dismutase
SOPC	1-Stearoyl-2-oleoylphosphatidylcholine

U. Agrawal • R. Sharma • S.P. Vyas (✉)
Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences,
Dr. H.S. Gour Vishwavidyalaya, Sagar 470003, MP, India
e-mail: uditaagrawal.pharma@gmail.com; vyas_sp@rediffmail.com; spvyas54@gmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_7

SOPS	Stearoyl-oleoyl-phosphatidylserine
STPP	Stearyl triphenyl phosphonium
TAT	Trans-activating transcriptional activator
tBHP	t-Butylhydroperoxide
TPP	Triphenylphosphonium
TPP	Triphenylphosphonium
VDAC	Voltage dependent anion channel
ZnO	Zinc oxide
ZnPc	Zinc phthalocyanine

7.1 Introduction

Targeting therapeutics to intracellular organelles of interest could be very effective in maximizing the drug effect and minimizing side effects. However, intracellular delivery and subsequent targeting to specific cellular compartments is challenging, especially for macromolecular drug delivery systems, due to a cell membrane that prevents their spontaneous entrance and that nanocarriers are taken up primarily by energy-dependent endocytosis process. Mitochondria are decisive cellular organelles known for their role in providing proficient energetic support through the chemiosmotic process of oxidative phosphorylation. The intrinsic pathway of apoptosis is controlled and governed by mitochondria by regulating the translocation of proapoptotic proteins from the mitochondrial intermembrane space to the cytosol and also participate in some forms of non-apoptotic cell death, such as necrosis.

In the context of drug delivery, effectiveness of any drug or gene therapy depends upon its delivery (site specific) within the cell (improve the efficacy) with the ability to reduce the toxicity. It is required to deliver drug to the specific cells as well as to inner cell compartments, which may contribute to a disease. Advancements in mitochondrial research and in medical technology have led to the fabrication of drugs that specifically target the mitochondria. Drugs that target mitochondria and exert their activity have become a focus of recent investigations due to their great clinical potential. The molecules, which selectively act on one or more mitochondrial sites for the palliative treatment and diagnosis of mitochondria associated diseases, are termed as "mitochondrial medicine" [1]. Nevertheless, targeting of mitochondria is particularly exigent as hurdles including biological barriers, additional barriers including intracellular diffusion/transport to the mitochondria and electrical potential across outer and inner mitochondrial membranes and toxicity affect the development of mitochondrial targeted therapeutics [2]. Moreover, processes such as the mobility of the system in the cytosol (because of the high concentrations of dissolved macromolecules), the rate of degradation in the cytosol and the rate of uptake into the targeted organelle limit intracellular targeting and thus affect targeting efficiency. Despite all these barriers and obstacles, drug delivery strategies are continuously being designed, and explored to target mitochondria specifically to make therapy more efficient and to minimize nonspecific side effects [3].

7.2 Importance of Mitochondrial Morphology

Mitochondria are typically elongated, thread-like organelles which differ from other organelles as they are double membranous structure with an unusual lipid composition. The mitochondrion is composed of four compartments each with different compositions, activities and functions: a porous outer membrane (lipid to protein ratio of 1:1), permeable to molecules smaller than about 6 kDa; an intermembrane space containing a number of specialized proteins; a convoluted and invaginated inner membrane containing the enzymes of oxidative phosphorylation and a series of metabolite carrier proteins; the mitochondrial matrix [4].

The mitochondrial matrix is the innermost space enclosed by the cristae membrane and contains enzymes of different breakdown pathways, including fatty acid oxidation, citric acid cycle, and the urea cycle, mitochondrial ribosomes, and specialized transfer RNAs as well as several copies of circular non-chromosomal mitochondrial DNA (mtDNA). mtDNA encodes 13 polypeptides, 22 tRNAs, and 2 rRNAs. All the 13 polypeptides are the components of mitochondrial enzymes. A number of transport proteins are present in the inner membrane—each of which is responsible for the transport of a specific ligand and compounds to the matrix space. For example, the ATP/ADP carrier (AAC) transferring ATP out of the matrix space, while simultaneously allowing ADP to cross the inner membrane. Mitochondria are found in all nucleated cells and are the principal generators of cellular ATP by oxidative phosphorylation. The number of mitochondria per cell is related to the energy requirements of the cell and varies according to the cell type, cell-cycle stage, proliferative state, and diseases related energy demands of the cell. Metabolically active organs such as liver, brain, cardiac and skeletal muscle tissues contain up to several thousands of mitochondria per cell while somatic tissues with low energy demands contain only a few dozen mitochondria [5]. Mitochondria have key implication in many pathways essential to both the life and death of cells. These organelles generate 80–90 % of ATP by oxidative phosphorylation needed for cell respiration and survival, regulate calcium flux, and have significant role in the integration of pro- and anti-apoptotic stimuli. Mitochondria cannot be formed de novo. They vary in shape, length, size and are dynamic organelles having complex, interconnected, and network like structures [6].

7.3 The Rationale for Mitochondria Drug Delivery

Mitochondria are the center of numerous fundamental metabolic pathways which are the prime target for pharmacological intervention. Cell's energy metabolism, regulation of programmed cell death and intracellular calcium concentration are controlled by mitochondria. Furthermore, the mitochondrial respiratory chain is the major source of damaging reactive oxygen species. Consequently, a number of

diseases including diabetes, cardiomyopathy, infertility, migraine, blindness, deafness, kidney, liver diseases, and stroke are the result of mitochondrial dysfunction. Somatic mutations in the mitochondrial genome contribute to aging, age-related neurodegenerative diseases as well as in cancer. Another concern is mitochondrial toxicity. A number of xenobiotics and therapeutics such as haloperidol and thiothixene exhibits mitochondrial toxicity by interfering with mitochondrial functions [7]. Membrane barriers as well as mitochondrial toxicity are significant limitations to be addressed for the effective mitochondrial therapeutics. In conclusion, the delivery of therapeutics into mitochondria may provide the basis for a large variety of future therapies. The natural aging process can be slowed down by delivery of antioxidants to the mitochondria. Mitochondrial DNA diseases may possibly be treated by the delivery of therapeutic DNA and RNA such as antisense oligonucleotides, ribozymes as well as plasmid DNA expressing mitochondrial encoded genes. The targeted delivery of drugs to mitochondrial-uncoupling proteins may be promising in obesity treatment. Designing therapeutic strategies specifically for killing cancer cells by exploiting their metabolic alterations may open up therapeutic possibilities for new anticancer therapies. Moreover, apoptosis-resistance of many cancer cells can be treated by delivering molecules which trigger apoptosis directly without involving mitochondria.

7.4 Mitochondrial Dysfunction and Related Disorders

Mitochondrial diseases include the disorders that are related to defects or absence of proteins that are utilized in mitochondria [8]. Dysfunctions caused by mutations mainly affect proteins of the respiratory chain and consequently normal energy production which can lead to severe diseases. Cancer, Neurological disorders such as Parkinson's, Alzheimer's, and Down syndrome are associated with mitochondrial dysfunction. Mitochondria also play a vital role in the pathology of many other diseases such as diabetes, amyotrophic lateral sclerosis, ischemic heart disease, hyperthyroidism, non-alcoholic fatty liver disease, and phenylketonuria. Mitochondrial dysfunction, which increases cellular glucose levels and consequently reduces insulin production [9] may cause diabetes. The maternally inherited type of mitochondria related diabetes is caused due to spot mutation/point mutation in one of the mitochondrial genes encoding tRNALeu (UUR). Amyotrophic lateral sclerosis is gradual and selective loss of motoneurons in cortex, brainstem, and spinal cord caused due to mutations in the enzyme superoxide dismutase 1 (SOD 1) in the mitochondria. Alzheimer's (AD) and Parkinson's disease (PD) are common age related diseases [10]. Patients with Alzheimer's disease showed altered activities of enzymes in tricarboxylic acid (TCA) cycle, mutations of mitochondrial fusion proteins, and accumulation of mtDNA mutations. The importance of mitochondria in the pathology of cancer and diabetes is discussed below:

7.4.1 Cancer

Cancer cell mitochondria are structurally and functionally different from their normal counterparts signifying the role of mitochondrial dysfunctions in the entire process of cancer development and progression. Synthesis of respiratory chain proteins due to mutations of the mitochondrial DNA (mtDNA) leads to increased electron leakage and ROS over production. Increased ROS level favors cell proliferation, DNA damage, genetic instability, resistance to antitumor agent's chromosomal instability, neoplasm metastasis, and carcinogenesis. Mitochondria regulate the intrinsic pathway of apoptosis by regulating the translocation of proapoptotic proteins from the mitochondrial inter-membrane space to the cytosol [11]. An intact outer membrane contains a variety of inactivated apoptotic proteins and once the integrity is lost; these apoptotic proteins are dumped into the cytoplasm and induce apoptosis. In cancer cells the antiapoptotic proteins are overexpressed with reduction in proapoptotic factors, enabling the cancer cells to be more resistant. In malignant lesions components of the permeability transition pore complex (PTPC) express and exhibit alterations, which control the exchange of metabolites and also mediate the permeability transition to trigger the release of cytochrome c. Thus, targeting of molecules or toxic substances which stimulate the permeability transition or alteration of mitochondria of tumor cells and devastate the mitochondrial DNA in mammalian cells to encourage apoptosis could be promising targeting strategy for cancer therapy [12].

7.5 Mitochondria-Targeted Agents Under Preclinical and Clinical Evaluation for Anticancer Therapy

Many of the compounds that target differences between mitochondria from normal cells and from cancer cells are currently under preclinical and clinical evaluation.

- Agents targeting the transition of cell metabolism
  Non-metabolically active glucose analog like 2-deoxyglucose inhibits glycolysis is currently under Phase I/II clinical trials. 3-bromopyruvate, an analog of lactic acid under preclinical testing, has shown beneficial property on tumor growth. Derivative of indazole-3-carboxylic acid, lonidamine (LND) inhibits glycolysis and improves the cytotoxicity of the doxorubicin and cisplatin is under multiple Phase III clinical trials. In addition, phloretin is known to sensitize cancer cells to daunorubicin for its anticancer activity and apoptosis to overcome drug resistance only under hypoxia. Another agent, dichloroacetate that inhibits the key enzyme pyruvate dehydrogenase kinase in cancer cells, is currently under Phase II clinical trials in brain tumor and some solid tumors.
- Agents targeting cellular damage caused by abnormal ROS production
  Several agents that increase ROS generation for cancer therapy are under clinical trials. Arsenic trioxide can cause an increase in electron leakage by interfering with

the OXPHOS, therefore promoting ROS generation, leading to cancer cell apoptosis. Redox-inactive vitamin E analog alpha-tocopheryl succinate is under Phase II study in melanoma, prostate cancer, colorectal cancer, mesothelioma, and breast cancer for anticancer activity.

The Phase I/II studies have demonstrated that 2-methoxyestradiol; an estrogen derivative that selectively kills human leukemia cells is well tolerated and causes disease stabilization in patients with solid malignancies or with multiple myeloma. Additionally, buthionine sulfoximine and imexon are both in Phase I clinical trials.

- Agents targeting the disabled apoptosis pathway
  Bcl-2, an antiapoptotic protein is a promising target molecule in cancer therapy. Antisense oligonucleotides specific for Bcl-2 RNA sequences (such as G3139) suppress particularly the proliferation of cancer cells or augment their sensitivity to chemotherapeutic drugs. G3139 and Gossypol, a BH3 mimetic is under Phase III clinical trials in addition with chemotherapeutic agents in a number of tumors. Peripheral benzodiazepine receptor (PBR) ligands, such as PK11195, RO5-4864 and diazepam, have shown antitumor effects both in vitro and in vivo, either as alone or in combination with other chemotherapeutic agents and have entered clinical trials. The promising results have been achieved in patients with recurrent glioblastoma treated with diazepam plus lonidamine.
- Agents targeting mutated mtDNA
  Several compounds that target mtDNA or enzymes related to its replication illustrate potential clinical applications. Among them, cisplatin, a classic anticancer drug in clinical use, is found to bind preferentially to mtDNA more than to nuclear DNA and shows higher cisplatin–mtDNA adduct levels, resulting in the inhibition of NADH-ubiquinone reductase and the decrease of ATP generation. Ditercalinium, a bis-intercalating agent accumulates in the mitochondria causing specific elimination of mtDNA and inhibition of its replication. In vitro studies have shown that vitamin K3 exhibits anticancer activity in breast and pancreatic cancer cells by specifically inhibiting the affect on DNA polymerase Υ, the mitochondrial enzyme responsible for mtDNA replication but its use as a potential anticancer agent remains to be evaluated.

7.6 Exploiting Mitochondrial Properties for Drug Targeting

Mitochondria exhibit some specific features that differ from other cellular compartments and also between normal and diseased mitochondria. These features can be exploited for developing a targeting strategy to transport biologically active molecules to and into the mitochondria within living mammalian cells. The distinct mitochondrial features, which govern the mitochondrial targeting strategies are:

- The high membrane potential across the inner mitochondrial membrane,
- The organelle's protein import machinery
- The mitochondrial fusion process

The first property that can be utilized for targeting is the mitochondrial membrane potential. Mitochondria are composed of a double membrane. Transmembrane electrochemical gradient is generated by the electron transport chain and ATP synthesis via oxidative phosphorylation that leads to a high membrane potential, negative inside and a pH difference, acidic outside [13]. Therefore, cationic molecules are attracted and preferentially taken up by mitochondria. The molecules get selectively accumulated in the mitochondrial matrix in response to their membrane potential. The inner mitochondrial membrane has to be crossed for the delivery of substances into the mitochondrial. Altogether, lipophilic as well as positively charged molecules can take the advantage of the membrane potential to accumulate in mitochondria [14]. Moreover, the outer membrane does not present a barrier to small molecules. These can basically diffuse through pores in the membrane formed by a membrane spanning protein.

Protein import pores the specific targeting sequences present in outer and inner membrane of mitochondria could potentially be utilized for transporting drug or DNA molecules to and/or into the matrix of mitochondria. In addition, any mitochondria-specific binding sites and unique protein receptor at the mitochondrial membranes could be exploited for drug targeting purposes. Mitochondrial targeting by mitochondrial fusion process so far has not been explored yet. Drug delivery using carriers might be a suitable approach to mitochondria targeting for drugs too large to pass the mitochondrial protein import pores.

7.7 Challenges of Targeting Mitochondria

Although mitochondria play an essential role in various significant pathologies, they have been an ignored target. It is due to the difficulty of selectively targeting molecules to this organelle in vivo. Various extracellular and intracellular barriers including cell membrane and the mitochondrial membrane impose formidable challenges to the drug delivery to mitochondria. The transportation through cell membrane is a prime requirement for drug delivery and therapy. For intracellular targeting a carrier system must cross the plasma membrane, a major barrier for large and charged molecules to enter the cytoplasmic space [15].

The challenge of targeting mitochondria by itself is to transfer the drug or drug carrier across two membranes in the case of mitochondrial matrix targeting. In targeting of the intermembrane space, it is sufficient to pass the outer mitochondrial membrane, but almost all potential targets are located in the mitochondrial matrix. Therefore, it is necessary to overcome both mitochondrial membranes which have only small pores and a highly lipophilic inner membrane. Theoretically, membrane impermeable probes can enter mitochondria through protein import pores (TOM and TIM complex), the pore protein porin, also referred to as the voltage dependent anion channel (VDAC), the mitochondrial permeability transition pore complexes (mPTPCs), through mitochondrial apoptosis-related channels and through apoptosis-related ceramide pores. But only the VDAC and the protein import pores are relevant

in normal mitochondrial function. The others are related to apoptosis and dysfunction and are localized in the outer but not the inner mitochondrial membrane.

Size and hydrophobicity of the molecules are the factors affecting and determining the diffusion rate through mitochondrial membrane. Hydrophobic (log P > 5) and relatively small molecular weight (500 mw) substances are transported through the plasma membrane by passive diffusion whilst hydrophilic molecules are screened off. However, different endosomal mechanisms are also utilized by the cell for the transport of macromolecules and carriers. All eukaryotic cells exhibit one or more forms of endocytosis. Many endogenous and exogenous ligands enter a cell by receptor mediated endocytosis. Optimal drug therapy not only depends on the delivery of bioactive molecule/macromolecule to its target cell but also its appropriate localization within that cell. Receptor targeting for selective uptake and internalization of drugs has further expanded with the introduction of new macromolecular drugs including DNA, peptides and proteins. The accessibility of sophisticated nanotechnology approaches to encapsulate drugs, providing controlled release capacity as well as protection of macromolecules from degradation prior to reaching the site of action, has provided an additional level of advantages. Macromolecules are primarily entrapped in endosomes followed by maturation to form late endosome and ultimately fusion with lysosome in order to exert its therapeutic effects [16]. The particles that do enter cells via the endosomal pathway, must escape the endosome before its fusion with the lysosome in order to prevent drug and carrier degradation. Due to the stability problems at endosomal/lysosomal pH macromolecular drugs such as proteins, peptides, DNA and drugs should bypass the endocytic pathway for their proficient delivery in the cytosol or other cellular organelles [17].

Different molecules which can be targeted to the mitochondria and pathways for mitochondrial targeting are represented in Fig. 7.1. Peptides namely cell penetrating peptides (CPPs) constitute a novel class of molecules capable of transferring molecules directly to the cytosol and bypassing endocytic pathway [16]. CPPs represent short polycationic sequences of about 10–30 amino acids which can extraordinary facilitate cellular gene/drug delivery. There are some more peptides that are structurally similar in that they all contain a short sequence of less than 20 amino acids with a positively charged arginine and lysine residues. This sequence is called "protein transduction domain" (PTD) and are considered to be significant to establish carrier cellular contact [18].

7.7.1 Cytosolic Barriers

The barriers that the molecules encounter before they reach mitochondrial organelle must be taken into account and special consideration should be given to diffusion of molecules from the plasma membrane through the cytosol. The cytosol has an active intracellular environment consisted of macromolecular species and specific components (water, ions and proteins) with minimal free space available [19].

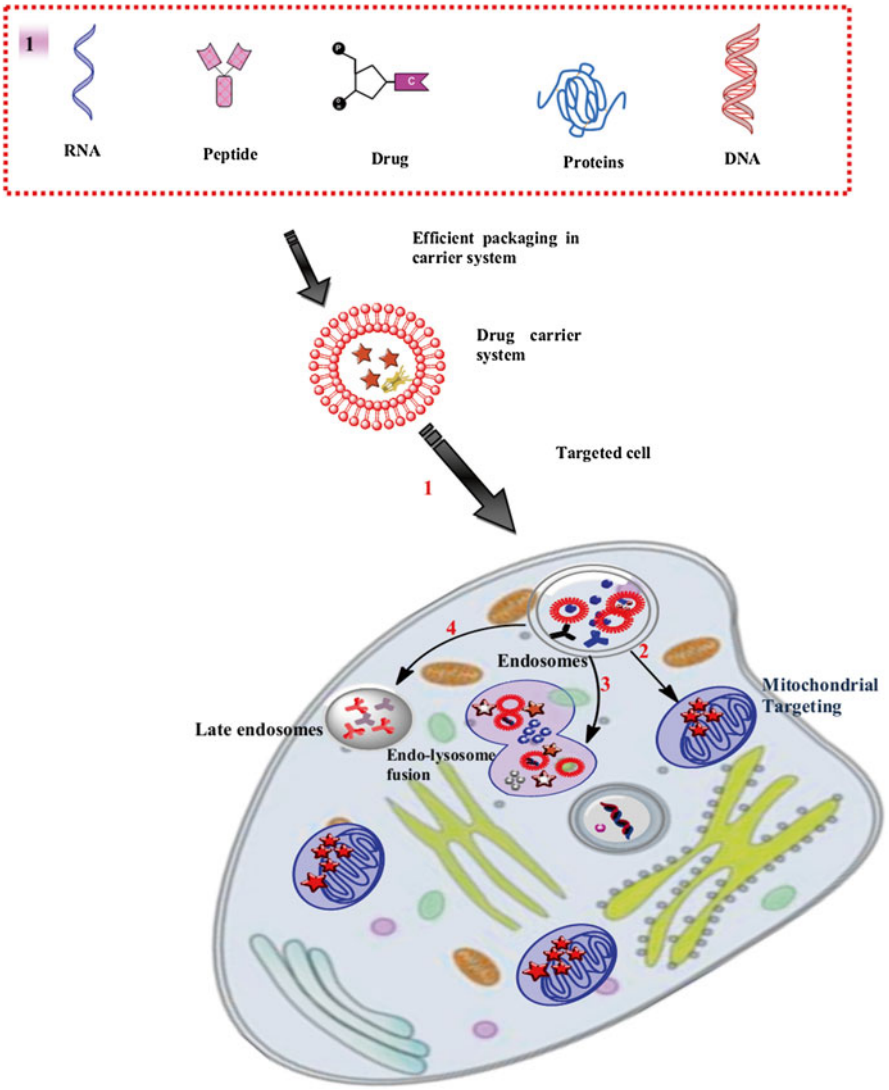

Fig. 7.1 (*1*) To design a successful drug delivery system, the therapeutic cargo must be encapsulated with therapeutic moiety depending on the physical characteristics, (*2*) intracellular trafficking of carrier including endosomal escape, (*3*) mitochondrial targeting, (*4*) showing the general route other than mitochondrial targeting

The high concentration of macromolecules (up to 400 mg/mL) [20] in the cytosol is referred to as molecular crowding, which constitute a significant diffusion barrier for molecules. Moreover, collisional interactions and binding to intracellular components in the cytoplasm are additional obstacles encountered during drug targeting to the mitochondria [21].

7.7.2 Mitochondrial Membranes

Mitochondria offer two anatomical barriers, the outer and inner mitochondrial membrane to the therapeutic agents to be targeted to the mitochondrial matrix. The outer mitochondrial membrane is weak barrier compared to the inner membrane and contains voltage-dependent anion channels (VDAC, also referred to porin) [22]. VDACs nonspecifically transport molecules into the transmembrane space between the outer and inner mitochondrial membranes and also coordinate with the mitochondrial permeability transition pore complex [23]. VDAC also integrates with PBR which primarily translocates cholesterol and benzodiazepine derivatives into the mitochondria for metabolism.

The outer membrane also contains mitochondrial protein transporters that translocate proteins which are generally positively charged and contain alpha helical secondary structure into the mitochondria [24]. The drug has to cross the inner membrane to reach the mitochondrial matrix. The inner membrane creates a strong negative membrane potential of approximately −180 mV due to the presence of electron transport chain which accounts for the higher accumulation of cationic lipophilic drugs than other molecules including hydrophilic, neutral, and anionic molecules. This negative membrane potential can be exploited to target therapeutics to this organelle. Mitochondrial accumulation of therapeutics involves factors such as electric potential, ion-trapping, and complex formation with cardiolipin [14]. It is reported that lipophilicity, charge and polar surface area of the drug influence mitochondrial uptake. Transporter proteins assist the uptake of other drug/protein molecules with low affinity for mitochondria. Integrating mitochondrial translocation ligands and/or positively charged ligands into the therapeutic carrier design may help targeted mitochondrial delivery [25]. Figure 7.2 represents schematic representation of biological barriers to mitochondria delivery and the approaches that could surmount the drug delivery challenges.

7.8 Mitochondria Targeting Strategies

A range of strategies for targeting bioactives to mitochondria has been explored. These strategies utilize biophysical properties of mitochondria, unique mitochondrial enzymes and targeting based on the transporter-dependent delivery to mitochondria. Compounds exhibiting or possessing a positive charge are attracted to mitochondria (due to the negative potential) but are unable to enter the mitochondrial matrix because of the impermeability of inner mitochondrial membrane to polar molecules. Therefore, additional physicochemical properties are required for mitochondrial targeting. Although it has been widely accepted that molecules should be comprised of positive charge and lipophilicity/amphiphilicity for mitochondrial targeting, the idea is still in controversies. Moreover, correlation between physicochemical properties and extent of accumulation in mitochondria could not be

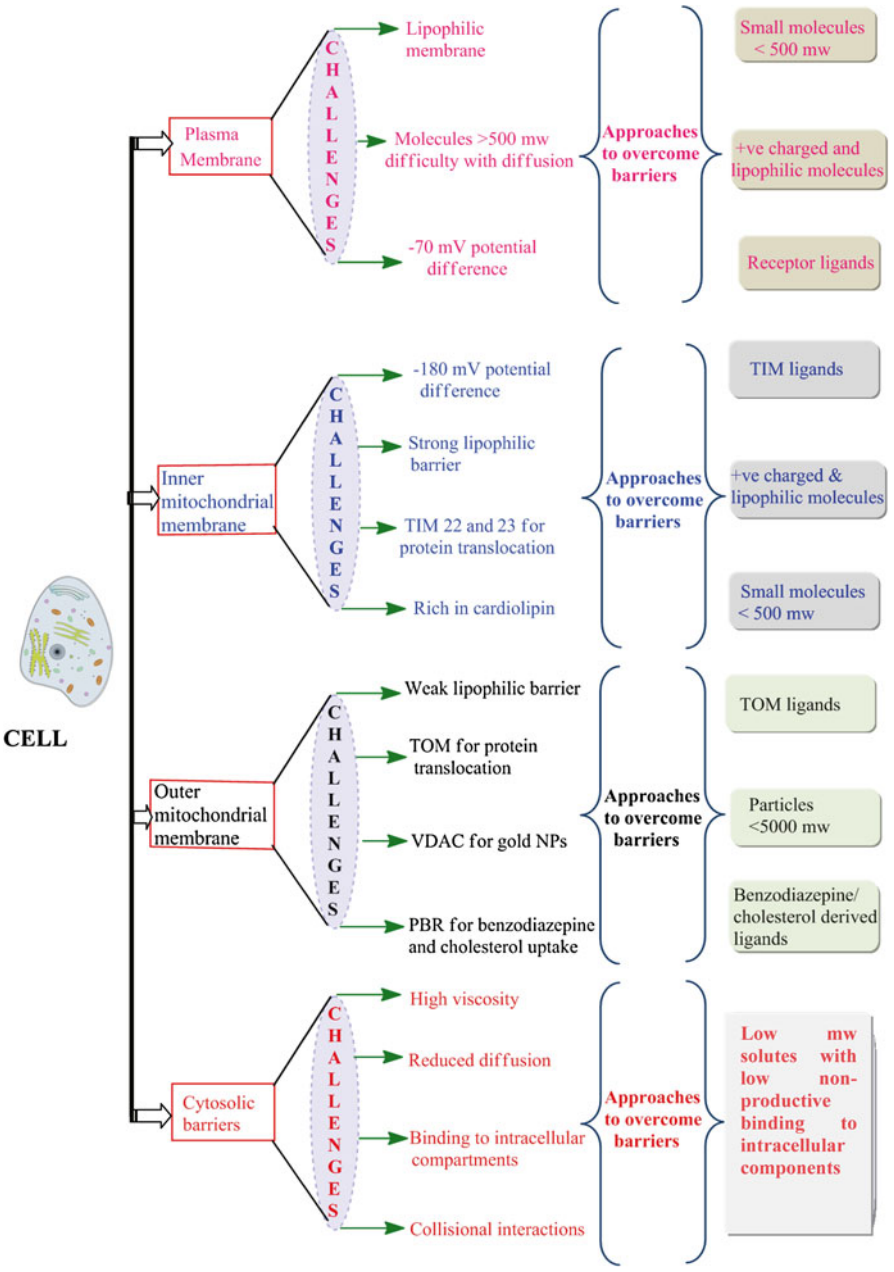

Fig. 7.2 Schematic representation of biological barriers for mitochondria delivery and the approaches to overcome the drug delivery challenges. Reproduced with permission from ref. [26]

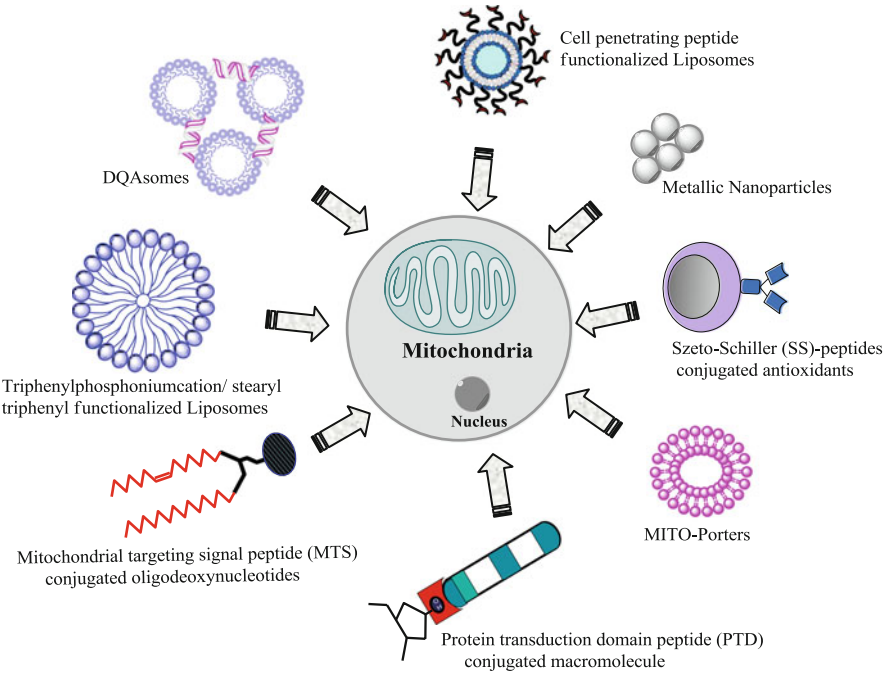

Fig. 7.3 Schematic representation of various mitochondrial drug targeting strategies

demonstrated [14]. However, several strategies directed/designed for mitochondrial targeting are shown to selectively deliver the drugs or molecules into mitochondria are discussed in this chapter. Figure 7.3 represents various targeting strategies for mitochondrial targeting.

7.8.1 Lipophilic Cation-Based Non-peptide Targeting Strategies

Lipophilic cations can easily transverse across the plasma membrane and the mitochondrial membranes because of the cationic charge and hydrophobic surface area, which in effect lowers the activation energy for their movement across the membrane. The negative membrane potential enables the positively charged moiety to move through membranes and accumulate in the mitochondrial matrix. Furthermore, lipophilic cations do not require a specific import mechanism. The mitochondrial membrane potential is about 180–200 mV which favors a 200- to 400-fold higher accumulation of lipophilic cation in the mitochondrial matrix. The membrane potential of the plasma membrane is about 30–60 mV, negative inside thus accumulation of these compounds in mitochondria relative to the extracellular environment is several fold higher [27].

Triphenylphosphonium (TPP) cation is best characterized and most widely used delocalized lipophilic cation for delivery to mitochondria, which was originally used to assess the mitochondrial membrane potential [28]. Uptake of TPP into mitochondria is well recognized and it is comparatively straightforward to introduce it into a compound late in the chemical synthesis scheme, typically by displacing a leaving group with triphenylphosphine cation [27]. Moreover, TPP or a methyl derivative of TPP does not require any receptor to penetrate into mitochondria. Drugs can be attached through the functional groups to the phosphorous atom or modified phenyl rings and carried into the matrix space [29]. It has been applied in various studies for mitochondrial targeting of antioxidants with an aim of protecting them from oxidative damage [30]. A wide range of antioxidants have been targeted to mitochondria by conjugation to the TPP lipophilic cation, including ebselen, lipoic acid, vitamin E, nitrones, plastoquinone, and nitroxides. This principle has also been successfully exploited to transport peptide nucleic acid (PNA) of 3.4–4 kDa by conjugating them with TPP for mitochondrial targeting in order to inhibit the replication of mutated mtDNA. Other lipophilic cations such as rhodamine 123 or tetraguanidinium oligomers are also found to accumulate selectively within mitochondria [31]. A mitochondria targeted analog of α-tocopherol (MitoE2), reduces iron/ascorbate-induced mitochondrial damage and neuronal and astrocytic cell death in pyramidal neurons. MitoQ exhibits antioxidant properties upon mitochondrial uptake. In mitochondria, MitoQ is reduced to ubiquinol, which is the antioxidant species that detoxifies reactive oxygen species (ROS), and is thereby oxidized to ubiquinone; the ubiquinone thus formed is reduced to ubiquinol.

7.8.2 Mitochondria-Targeted Peptides

Studies describe a variety of peptide and amino acid based mitochondrial transporters which are designed to utilize charge-driven uptake into the mitochondria. These peptides appear to enter cells via direct mode of uptake, avoiding endosomal and/or lysosomal sequestration that would prohibit their ability to accumulate in mitochondria [32]. Peptides with antioxidant properties present a different mitochondria targeting strategy.

The peptide sequences are resistance to hydrolysis and, have considerable pharmacokinetic properties. The observed cell permeability of peptides is dependent on charge and lipophilicity. Szeto-Schiller (SS)-peptides (positively charged peptides) is an approach which could be used for mitochondrial targeting. The structural motif consisted of four alternating aromatic and basic amino acids and possesses three positive charges at physiological pH. Studies have revealed their rapid uptake through the plasma membrane and accumulation in mitochondria in isolated cells [33]. The uptake of Szeto-Schiller peptides into mitochondria does not depend upon the negative membrane potential and the mechanism behind their uptake is currently not clear [34]. The novel approach for targeted delivery of antioxidants to the inner mitochondrial membrane using Szeto-Schiller (SS) peptide antioxidants resulted in reduced ROS and cell death caused by t-butylhydroperoxide (tBHP) in

neuronal N2A cells (EC50 in the nM range). These have been investigated in a variety of in vitro and in vivo disease models.

The peptides inhibit lipid peroxidation by scavenging hydrogen peroxide and peroxynitrite. Tyrosine and dimethyltyrosine residues may produce antioxidant action. SS peptide antioxidants inhibit the permeability transition (PT) and swelling, and reported prevented cytc release induced by Ca^{2+} in isolated mitochondria. Because ROS and PT have been implicated in myocardial stunning these peptide antioxidants can notably progress contractile force in an ex vivo heart model. Therefore, it can be speculated that these peptidal antioxidants can be effective anti-aging treatment and diseases associated with oxidative stress [33].

Mitochondria-penetrating peptides (MPPs) are one more promising delivery vectors for specific and effective mitochondrial transport [35]. These consist of four or eight alternating positively charged, hydrophobic, and partly unnatural amino acids. Small molecules, biotin and trolox, a water soluble analog of vitamin E are delivered by MPPS into mitochondria [32]. Use of cell penetrating peptides (CPP) is another approach which is able to transport the cargoes of much higher molecular weight compared to their own [36]. CPP are larger peptides consisting of up to 30 positively charged or alternating positively charged and hydrophobic amino acids. Studies show that number of molecules such as proteins, peptides, nucleic acids, and even nanocarriers like liposomes can be delivered in this way into cells [37]. Natural mitochondrial leading sequences (MLSs), derived from mitochondrial proteins are competent to deliver molecules into mitochondria. They are synthesized in the cytosol, with 10–80 amino-terminal pre-protein to be translocated to their ultimate mitochondrial destination. These are positively charged, hydrophobic, and hydroxylated amino acids with an ability to form an amphipathic α-helix that presents one positively charged surface and one hydrophobic surface. These structural characteristics are imperative for the identification by the mitochondrial protein import pores and therefore when an MLS is attached to a non-mitochondrial protein, it can specifically direct the protein into mitochondria [38].

Most of these proteins are encoded in the nuclear genome and consequently delivered to mitochondria by mitochondrial targeting signal peptide (MTS) that is located at the N-terminus of the precursor protein [39]. These MTSs are typically consisting of 10–70 amino acids, which are removed in 1 or 2 proteolytic steps once delivered to the mitochondria. These MTS peptide can be used to deliver proteins to the matrix of the mitochondria. The MTS sequence leads the cargo protein to the mitochondria, and is then cleaved, allowing for the absolute localization and function of the fused protein [40].

Cyclosporin A (CsA) is a cyclic peptide that inhibit mPTPC formation and delays cell death caused by oxidative stress, and hence used on protecting the heart and the brain from ischemia–reperfusion injury. Although CsA is a potential drug for an anti-ischemic drug, it is difficult to predict the concentrations of CsA in mitochondria as there are additional targets of CsA in the cell. Therefore to achieve therapeutic benefit of CsA, mitochondria-specific drug carrier system should be developed [41].

7.8.3 *Lipidic and Polymeric Nanocarriers for Mitochondrial Targeting*

Nanocarriers offer numerous advantages in the design of therapeutics to overcome the challenges and limitations associated with mitochondrial targeting. Nanocarrier loaded with drug can be conjugated with organelle specific targeting moieties. Targeting moieties include peptide sequences and non-peptide molecules that can be recognized and interact with the mitochondrial membranes [42]. Mitochondrial delivery of drug requires escape of carrier from endosomes after endocystosis. The drug delivery system decorated with targeting moieties has to release the drug into the cytosol for the intracellular organelle specific targeting (Table 7.1). Liposomes, nanoparticles, micelles and multifunctional nanoparticles have been proposed for the delivery of hydrophobic drugs to various sub-cellular organelles including mitochondria. In a study fluorescently labeled micelles were found to be distributed through several cytoplasmic organelles including a majority of them being associated with the mitochondria. It was found that internalization of drug incorporated in micelles was better than the free drug [54].

It is demonstrated that the quantum dots that are enfolded in micelles and conjugated to a mitochondrial targeting peptide could preferentially accumulate in mitochondria when they are applied to the intact cells [55]. Another strategy that can be explored to target mitochondria is the utilization of water soluble fullerene derivatives that accumulate in mitochondria as well [56]. Moreover, drug conjugates of HPMA copolymer are also possible candidates which can be explored for mitochondrial targeting. Drug conjugates were synthesized using a photosensitizer mesochlorine 6 (Mce 6). Mitochondrial targeting of HPMA copolymer-bound Mce 6 enhanced cytotoxicity as compared to non-targeted HPMAcopolymer-Mce6conjugates [57].

7.8.4 *Bolasomes*

Dequalinium (DQA) is a cationic bolaamphiphile with delocalized charge centers. It forms liposome like aggregates in water called DQAsomes/bolasomes that act as a vector for the transportation of DNA to mitochondria in living cells. DQAsomes exhibited higher mitochondrial accumulation and retention. Positively charged DQAsomes are attracted towards the negatively charged mitochondrial transmembrane of the tumor cells. García-Pérez et al. [58] reported that DQA showed anticancer activities and induced a concentration-dependent oxidative stress by decreasing glutathione (GSH) level and increasing ROS in a cell type specific way. Inhibitors of the JNK and p38 stress regulated kinases potentiate DQA-induced NB4 cell death indicating a protective function for these enzymes.

DQA encapsulating paclitaxel exhibited enhanced apoptosis activity over unencapsulated paclitaxel in COLO25 cells [59]. They are capable of increasing the

Table 7.1 List of various nanosystems designed for targeted mitochondrial delivery for the treatment of carcinomas

Nanocarrier/ therapeutic moiety	Disease targeted	Mechanism	Results	Ref.
Liposomes modified with TPGS1000-TPP/ Paclitaxel	Resistant lung cancer	Enhancing the apoptosis by acting on the mitochondrial signaling pathways.	The TPGS1000-TPP conjugated liposomes facilitated the mitochondrial targeting and uptake. The targeting liposomes could induce the apoptosis of the drug-resistant lung cancer by releasing cytochrome C, and initiating a cascade of caspase 9 and caspase 3 reactions as well as cause the apoptosis by activating the pro-apoptotic Bax and Bid proteins, and suppressing the anti-apoptotic Bcl-2 protein.	Zhou et al. [43]
Lonidamine liposomes in combination with targeting epirubicin liposomes/ Lonidamine and epirubicin	Drug-resistant cancer	Enhancing the cytotoxic effect by acting on the mitochondrial signaling pathways.	The targeting lonidamine liposomes significantly enhanced the inhibitory effect of the targeting epirubicin liposomes in the drug-resistant A549cDDP cells in a lonidamine dose-dependent manner. The efficacy in treating the drug-resistant A549cDDP xenografted tumor model after administration of the targeting lonidamine liposomes plus targeting epirubicin liposomes was the most significant	Li et al. [44]
Liposomes/Topotecan	Drug-resistant breast cancer and inhibiting invasive metastases of melanoma	Dissipated mitochondrial membrane potential, opening of mitochondrial permeability transition pores, release of cytochrome C, and activation of caspase 9 and 3.	The targeting liposomes had stronger inhibitory effect on the resistant tumor spheroids in vitro, enhanced accumulation in resistant MCF-7/ADR cell xenografts in mice, as well as being very effective on resistant MCF-7/ ADR cell xenografts in mice, and having a marked anti-metastatic effect on the naturally resistant B16 melanoma metastatic model in mice	Yu et al. [45]
Folic acid and TPP decorated Liposomes mitocancerotropic (FA-MTLs)/ Doxorubicin	Cancer cells	Redox cycler—doxorubicin acts as a source of exogenous ROS production	Mitocancerotropic liposomes showed superior activity over mitochondria targeted liposomes which confirm the synergistic effect imparted by the presence of dual ligands as folic acid and TPP on the enhancement of cellular and mitochondrial delivery of doxorubicin in KB cells.	Malhi et al. [46]

Liposomes modified with novel TPP-PEG-PE conjugate/ Paclitaxel	Cancer	Apoptosis	TPP-PEG-PE modified liposomes demonstrated efficient mitochondrial targeting in cancer cells as shown by confocal microscopy in colocalization experiments with stained mitochondria. The system demonstrated enhanced PTX induced cytotoxicity and antitumor efficacy in cell culture and mouse experiments	Biswas et al. [26, 47]
Liposomes modified with STPP/Paclitaxel	Ovarian carcinoma	Decrease in mitochondrial membrane potential and related to caspase-independent cell death	Incorporation of paclitaxel in STPP liposomes results in improved accumulation of paclitaxel in mitochondria as well as improved cell kill in the Ovcar-3 paclitaxel resistant cell line.	Solomon et al. [48]
Liposomes modified with (DQA-PEG2000-DSPE conjugate/Resveratrol	Intrinsic multidrug resistance (MDR) of cancers	Apoptosis	In conclusion, mitochondrial targeting resveratrol liposomes would provide a potential strategy to treat the intrinsic resistant lung cancers by inducing apoptosis via mitochondria signaling pathway.	Wang et al. [49, 50]
CTAB functionalized Au nanorods/Gold	Cancer	Apoptosis	Au nanorods have distinct effects on cell viability via killing cancer cells. Obvious differences in cellular uptake, intracellular trafficking, and susceptibility of lysosome to Au NRs by different types of cells resulted in selective accumulation of Au NRs in the mitochondria of cancer cells.	Wang et al. [49, 50]
STPP functionalized liposomes/Sclareol	Colon cancer and leukemia	Apoptosis	STPP functionalized liposomes exhibited 160 % increase in apoptotic events in COLO205 cells and presented caspase-8 activity and 300 % increase in caspase-9 activity.	Patel et al. [51]
Proteoliposomes/Bak	Cancer protein therapy	VDAC play a role in apoptosis and Bak induces mitochondrial membrane permeability upon activation	Membrane proteins integrated in natural liposomes can represent an excellent candidate for cancer protein therapy	Liguori et al. [52]

(continued)

Table 7.1 (continued)

Nanocarrier/ therapeutic moiety	Disease targeted	Mechanism	Results	Ref.
MITO-Porters (Liposome surface modified with octaarginine)/GFP	Cancer and neurodegenerative disorders	MITO-Porter promote fusion with the mitochondrial membrane and release of its cargo to the intra-mitochondrial compartment in living cells	The MITO-Porter holds promise as an efficacious system for the delivery of both large and small therapeutic molecules into mitochondria.	Yamada et al. [53]
STPP functionalized Liposomes/Ceramide	Cancer	Targets cytochrome c release, ROS production, and apoptosis	The in vitro CLSM demonstrated that STPP functionalized liposomes localized within the mitochondria of 4T1 breast cancer cells and induced more apoptosis, based on a characteristic DNA ladder and indicative of apoptosis. The STPP functionalized liposomes treated mice group survived until day 18 and dose was 6 times less than the typical dose.	Boddapati et al. [54]

paclitaxel accumulation in the mitochondria. Apoptotic activity of paclitaxel was enhanced following the mitochondria-specific delivery at concentrations, at which the free drug does not have any significant cytotoxic effect. Paclitaxel loaded DQAsomes have led to an improved ability to inhibit the growth of human colon cancer tumors in nude mice. Decoration of DQAsomes containing paclitaxel with folic acid further improved the antitumor efficiency [60]. The folate receptor is over expressed in a large variety of human tumors. Conjugated nanocarriers are internalized in a tumor cell-specific manner through folate receptor-mediated endocytosis resulting in an increased toxicity of the encapsulated drug. Folic acid conjugated DQAsomes were studied for cell cytotoxicity using HeLa cells possessed improved antitumor activity as compared to plain paclitaxel loaded DQAsomes. They are found to be better delivery systems as they could deliver the drug not only to the cytosol but also to mitochondria whereas folic acid conjugated liposomes delivered the drug into the cytosol only [59]. Figure 7.4 shows untreated cells, cells incubated with empty DQAsomes and cells exposed to naked pDNA, respectively.

DQAsomes complexed with plasmid DNA can transport and release nucleic acid into mitochondria after interacting with mitochondrial membrane [60]. In a study DNA conjugated to mitochondrial targeting sequences (MLS) was entrapped into the DQAsomes and successfully used to deliver the DNA into the mitochondria [61]. Vaidya et al. [62] studied the antitumor activity of folic acid conjugated

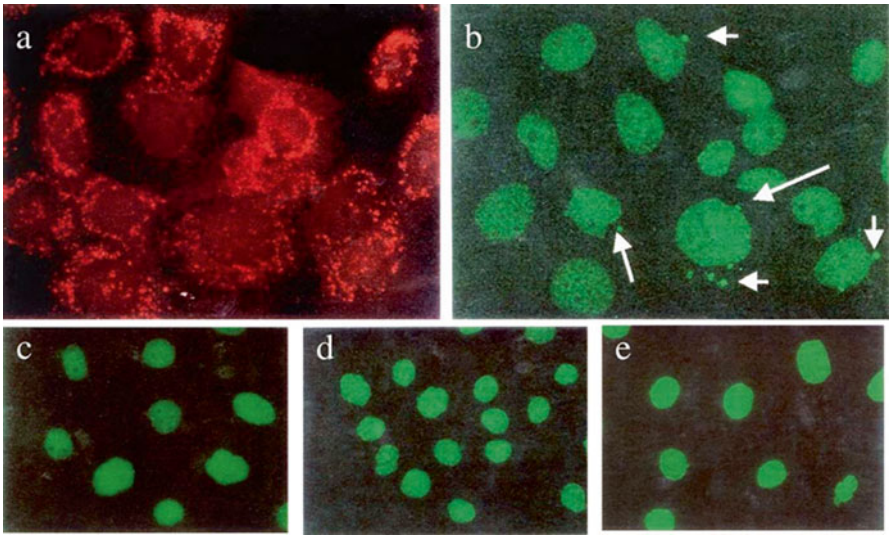

Fig 7.4 Fluorescence microscopic images of BT20 cells exposed to DQAsome/pDNA complex. The cells were incubated with MitoTracker Red CMXRos for staining mitochondria and with SYBR Green I for staining free DNA. *Top row*: Cells + DQAsome–pDNA; (**a**) MitoTracker, (**b**) SYBR. *White arrows* indicate plasmid DNA transported by DQAsomes to the site of mitochondria and released from the DQAsome–DNA complex upon contact with the mitochondrial membrane. *Bottom row*: Controls, stained with SYBR. (**c**) Untreated cells; (**d**) cells + empty DQAsomes; (**e**) cells + naked pDNA. (Reproduced with permission from D'souza et al. [60])

DQAsomes using HeLa cells. It was revealed that folic acid conjugated DQAsomes show improved antitumor activity as compared to un-conjugated DQAsomes, folic acid conjugated liposomes and paclitaxel solution.

7.8.5 Lipidic Nanocarrier-Liposomes

Liposomes are the most potent and investigated nanocarrier system for mitochondrial targeted therapeutics because of their biocompatibility and safety. Proteoliposomes prepared by incorporating a crude mitochondrial membrane fraction into liposomes was the first research that signifies the utility of liposomes for targeting mitochondria [63]. Liposomes can be fused with mitochondrial membranes releasing their drug load into mitochondria. This approach takes benefit of the fact that mitochondria are able to fuse with one another [64].

Cationic liposomes made up of DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and DOTAP (dioleoyl-1,2-diacyl-3-trimethylammoniumpropane) were used to deliver pro-apoptotic peptide D-(KLAKLAK)2 together with an antisense oligonucleotide into mitochondria of the cell in order to treat cancer [65]. Conjugation of a lipophilic cationic ligand to the liposomes could target drugs to the mitochondria. In a study ceramide (anticancer agent that targets ROS production) loaded liposomes functionalized with stearyl triphenyl phosphonium (STPP) were developed and assessed for antitumor efficiency [54]. STPP shows both cationic and lipophilic properties. STPP functionalized liposomes were localized within the mitochondria of 4T1 breast cancer cells and found to induce more apoptosis in 4T1 breast cancer cells, compared to unconjugated liposomes. Survival rates of tumor induced BALB/c mice models were amplified as compared to non-targeted liposomes or no treatment control since the drug was targeted to the mitochondria [54]. Further, STPP functionalized liposomes enhanced the efficacy of sclareol against colon cancer and leukemia. These liposomes were found to enhance the apoptosis, caspase-8 activity, and caspase-9 activity in COLO205 cells compared to non-targeted liposomes. The amount of drug required for an effective therapeutic response by functionalized liposomes was less compared to non-functionalized liposomes. It is also reported that STPP-liposomes directed successful accumulation of rhodamine labeled phosphatidylethanolamine into mitochondria of live cells [51].

MITO-Porter is a liposomal carrier system with octaarginine modified at the surface. It promoted fusion with mitochondrial membranes and was shown to specifically deliver contents into mitochondria. Macropinocytosis rather than clathrin-dependent endocytosis is the main mechanism of internalization of MITO-Porter in the cells [3]. MITO-porter liposomes have also been used to deliver green fluorescent protein [66] as well as propidium iodide [67] to mitochondria suggesting their efficacy in delivery of small and large molecules to mitochondria. Moreover, MITO-Porter was modified with R8 peptide, which mimics the trans-activating transcriptional activator (TAT) and was found to function as a useful moiety for cellular uptake as well as mitochondrial targeting [68].

The multifunctional envelope-type nano-device (MEND) permits efficient and simple packaging of plasmid DNA, proteins or other macromolecules for gene delivery. It consists of lipid envelope equipped with various functional devices to mimic envelope-type viruses [53]. MEND is based on the new packaging concept 'programmed packaging', is similar to envelope-type viruses, comprises a condensed core, such as plasmid DNA, and a lipid envelope equipped with various functional devices, which keep their unique basic properties and exist as separate structures in the compound. The merits of MEND include enzymes protection, improved packaging efficiency, controlled size and release over a short period of time. To locate these nanoparticles selectively in the mitochondria of cancer cells, different strategies like peptides for endosomal escape, ligands for specific receptors and mitochondrial targeting drugs could be easily incorporated into the core and onto the surface of particles. These unique characteristics make this design a capable module for drug delivery [69].

Lipoplexes are small partially stable particles formed by mixing of nucleic acids with cationic liposomes which protect nucleic acid from nuclease degradation and also enhance cellular transfection. They also facilitate nucleic acids release from the intracellular vesicles before they reach the destructive lysosomal compartments [70]. Lipofectin is a cationic liposome that is commercially available as a transfection reagent used for intracellular delivery of DNA [71]. It is composed of the cationic lipid 2,3-bis-(oleoyl)oxipropyl-trimethyl ammonium chloride (DOTMA) and di-oleoylphosphatidylethanolamine (DOPE). A spontaneous electrostatic interaction between the negatively charged DNA and the positively charged liposomes results in condensation of the nucleic acids. Moreover, the resulting cationic liposome/DNA complexes display a net positive charge that is important for their association with the negatively charged cell surface. Intracellular release of complexed DNA is facilitated due to the fusogenic properties of the cationic liposomes based formulation that can induce fusion and/or destabilization of the plasma membrane. Intact DNA can be delivered into the cytoplasm by the virtue of cationic liposomes. The DNA released into the cytosol consequently move to the mitochondrial matrix through protein import machinery if DNA is conjugated with MLS. DNA is first conjugated with the MLS and this complex is entrapped within the cationic liposomes. The entrapped DNA is released from the liposomes in to the cytosol; subsequently DNA is carried by the MLS peptides to the mitochondrial matrix (Fig. 7.5).

7.8.6 Polymer Nanocarriers

Polymeric nanoparticles are the system of choice because of their stability, biodegradability, and biocompatibility. Moreover, polymeric nanoparticles can be chemically conjugated and modified to targeting ligands and/or drugs. Poly-lactide-co-glycolide (PLGA) is nontoxic and biocompatible polymer most commonly explored for drug delivery applications. PLGA nanoparticles loaded with superoxide dismutase (SOD), was found to be more efficacious in preventing of H_2O_2

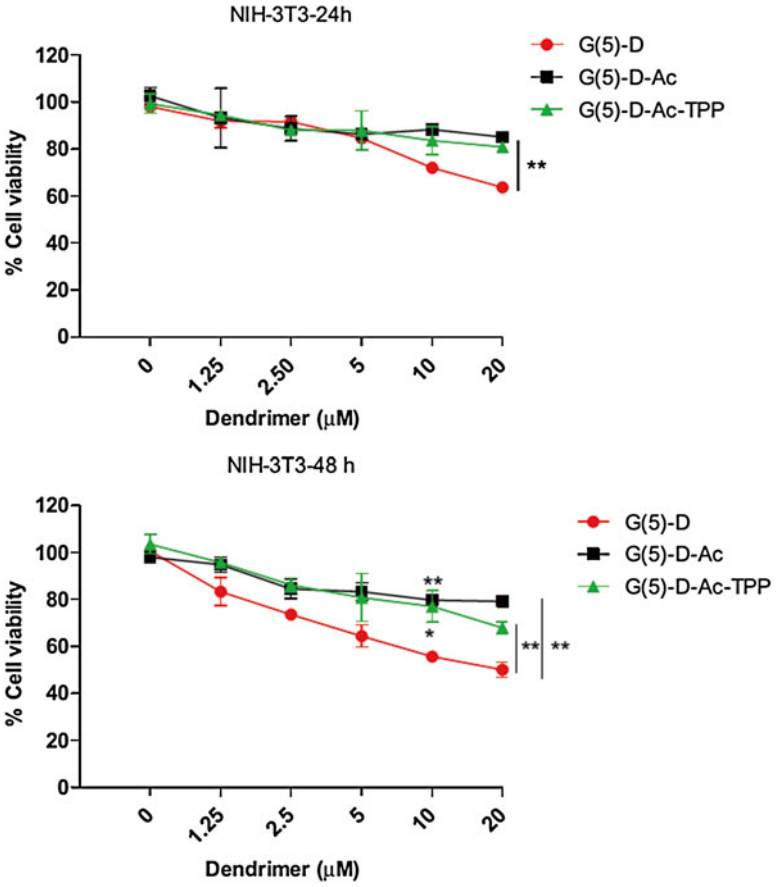

Fig 7.5 Dose-dependent cytotoxicity of dendrimers against NIH-3T3 cells at 24 and 48 h. Data are expressed as mean SD of three experiments carried out in triplicate. Difference between G(5)-D and G(5)-D-Ac/G (acylated dendrimer) (5)-D-Ac-TPP (TPP-anchored dendrimer) were analysed. (Reproduced with permission from Biswas et al. [26, 47])

induced neuronal cell death compared to SOD alone and SOD conjugated to polyethylene glycol [72]. These nanoparticles were fabricated using a w/o/w double emulsion method and it was speculated that the H_2O_2 produced by SOD as a result of ROS reduction, depletes intracellular antioxidants such as catalase, which neutralize H_2O_2. The study demonstrates the potential of antioxidant loaded polymeric nanoparticles in protecting cells from oxidative stress induced cell death. Marrache et al. [73] successfully induced immune response through mitochondria-targeted biodegradable polymeric nanoparticle containing zinc phthalocyanine (ZnPc) (photosensitizer).

Multifunctional nanoparticles are investigated to provide a promising method for mitochondria-targeted cancer treatment. Several multifunctional mitochondrial nanoparticles have been effectively used for cancer therapy. For example,

mitochondrial photo damage upon photo-irradiation by dendrimer phthalocyanine-encapsulated polymeric micelles and αvβ3 integrin-targeting by ligand conjugated doxorubicin (DOX)-micelles can increase DOX mitochondrial accumulation [74]. Biswas et al. [26, 47] conjugated mitochondriotropic ligand triphenylphosphonium (TPP) on the surface of the poly(amidoamine) (PAMAM) dendrimer. The newly developed TPP-anchored dendrimer (G(5)-D-Ac-TPP) was efficiently taken up by the cells and demonstrated good mitochondrial targeting. In vitro cytotoxicity experiments carried out on normal mousefibroblast cells (NIH-3T3) showed greater cell viability in the presence of the G(5)-D-Ac-TPP compared to the parent unmodified G(5)-D. To assess the effect of surface modification on the cytotoxicity, a dose dependent cell viability experiment with all three dendrimers was performed in normal mousefibroblast cell line (NIH-3T3) at 24 and 48 h incubation periods. The results demonstrated that the modified dendrimers were significantly less toxic than the starting material G(5)-D (Fig. 7.5).

7.8.7 Metal Nanoparticles

Mitochondrial targeting can be successfully executed by nanoparticles prepared from metal elements including gold, platinum and titanium dioxide. They have distinctive properties including smaller size (<10 nm), antioxidant capabilities, and ease of attachment of targeting ligands. Bimetallic nanoparticles may act as the delivery vehicle and therapeutic agent and are also capable of acting as antioxidant species. Conjugation of targeting ligands (peptides, proteins or nucleic acids) to these bimetallic nanoparticles may be a useful approach to target the mitochondria. However, the cellular toxicity of these nanoparticles is yet to be tested. Different types of metal nanoparticles are discussed below. Gold nanoparticles have been prepared with various sizes from <10 nm to approximately 100 nm [75]. Surface of the gold nanoparticles generate a magnetic field upon laser irradiation due to plasmon resonance. Altering this surface of the nanoparticle with proteins or DNA alters their surface plasmon resonance properties. This property has been used in development of novel functional drug delivery systems, diagnostic systems [76], sensitive biosensors for determination of protein–ligand binding reactions, biosensing assays [77]) and micro-electromechanical systems. Only very few studies allude to the potential application of these devices as novel mitochondrial targeting systems.

Gold nanoparticles are known to have detrimental effects on the mitochondrial as they are found to disrupt the integrity of the outer mitochondrial membrane and release of cytochrome c from the mitochondrial electron transport chain, which is known to cause cell death [22]. It is reported that gold nanoparticles of 3 nm could permeate the outer mitochondrial membrane of heart mitochondria whereas nanoparticles of 6 nm were not able to permeate. Therefore, the delivery of gold nanoparticles is size dependent whereby particles smaller than or equal to 3 nm are capable of crossing the outer mitochondria membrane. It was found that nanoparticles entry into the mitochondrial is dependent on VDAC as the entry is inhibited

by VDAC inhibitors. This pathway is of therapeutic value as it is involved in cellular apoptosis as well. Surface charge and nanoparticle concentration are important determinants other than size as they govern cellular toxicity [78]. Further, toxicity was characterized by lysis of anionic 1-stearoyl-2-oleoylphosphatidylcholine (SOPC)/stearoyl-oleoyl-phosphatidylserine (SOPS) liposomes and neutral SOPC liposomes. Cationic gold nanoparticles lysed tenfold more anionic liposomes (about 20 %) than anionic gold nanoparticles (about 2 %) in 5 min. Lysis of neutral liposomes (about 15 %) was nearly the same as for anionic and cationic gold nanoparticles at 5 min; however, anionic gold nanoparticles lysed neutral liposomes more (about 5 %) compared to anionic liposomes (about 2 %) at 5 min. In addition to the diameter of the gold nanoparticles, the charge of the nanoparticle and concentration also contribute to cellular toxicity. Although gold nanoparticles exhibit toxicity, they also exhibit therapeutic effects.

Surface modified chitosan functionalized gold nanoparticles were found to be more proficient at eliminating ROS in an $H_2O_2/FeSO_4$ system than ascorbic acid [79], and the antioxidant activity was further augmented with an increase in chitosan concentration. However, the activity was independent of size of gold nanoparticles. Although the results suggest that chitosan may be responsible for the antioxidant activity when complexed with gold, chitosan has no known antioxidant effects and may exhibit cytotoxicity [80].

Gold nanoparticles functionalized with polyamidoamine (PAMAM) dendrimers are capable of acting as antioxidants by reducing ROS to water and oxygen [81]. PAMAM dendrimer functionalized gold nanoparticles were fabricated by reducing the $HAuCl_4$–dendrimer mixture with $NaBH_4$ under stirring for 30 min. The gold nanoparticle may contribute to the antioxidant effects of dendrimer functionalized gold nanoparticles as PAMAM dendrimer alone was not capable of eliminating free hydroxyls. PAMAM functionalized gold nanoparticles with terminal carboxyl groups had a rate constant 85 times faster than ascorbic acid. The dendrimer functionalized gold nanoparticles showed smaller size than chitosan-gold nanoparticles hence may be more capable than the chitosan-gold nanoparticles. They also have slightly higher efficiency at eliminating ROS. The gold nanoparticles can be used to functionalize and provide for mitochondrial targeting of the dendrimer along with the drug content.

A study demonstrated preferentially induced cell death in cancer cells, but not in normal cells by gold nanorods functionalized with CTAB (cetyltrimethylammonium bromide, a cationic lipophilic molecule). They accumulated in the mitochondria of cancer cells, but not in the mitochondria of normal cells [49, 50]. CTAB is known to induce toxicity and may be partially responsible for cell death induced by CTAB functionalized gold nanorods [82]. Preferential uptake of cationic CTAB functionalized gold nanorods may be attributed to the extremely negative membrane potential across the inner mitochondrial membrane. Mitochondrial membrane potential disruption induced cancer cell death as determined by formation of ROS by flow cytometry and a JC-1 dye assay. Cancer cells had significantly higher levels of ROS than non-cancerous cells with gold nanorods. Gold nanoparticles conjugated to TPP are under investigation for their possible benefits as targeted delivery

option. Further, these studies demonstrate that surface modification of gold nanoparticles enhances mitochondrial delivery. Gold nanoparticles elicit anticancer therapeutic effect in the absence of drug. Gold nanoparticles have the ability to act as drug carrier as well as an antioxidant, which make them an attractive nanoparticle for mitochondrial delivery.

Titanium dioxide particles have been also investigated as targeted nanotherapeutics to enter the mitochondria for gene regulation control [25]. Surface of the nanoparticle modified with mitochondrial-specific oligonucleotides exhibit their mitochondrial specificity. It is reported that the dopamine complex with TiO_2 nanoparticles is much more stable than the glycidyl isopropyl ether coating, which is also stable in the presence of sunlight. Intense red color was generated due to the charge transfer between dopamine and TiO_2 that confirms the conjugation of the dopamine labeled oligonucleotide to the nanoparticle. Oligonucleotide functionalized TiO_2 nanoparticles were applied to MCF-7/WS8 breast cancer cells and were subjected to electroporation as a transfection tool. TEM images revealed the accumulation of nanoparticles within the mitochondria of the cell. The authors also confirmed mitochondrial targeting in a rat pheochromocytoma cell (PC12) line. These results demonstrated that mitochondrial intracellular targeting is possible with nanoparticles coated with oligonucleotides specific to the mitochondrial DNA. Interestingly, targeted mitochondria delivery is achieved by non-functionalized titanium dioxide particles of 5–23 nm with minimal nuclear delivery [83]. However, the mechanism by which the oligonucleotide conjugated nanoparticle is endocytosed and reached the mitochondria is not clear. Since larger titanium dioxide particles (5,000 nm) were unable to enter mitochondria, mitochondrial delivery appears to be affected by nanoparticle size and probably also by nanoparticle materials since titanium dioxide can enter mitochondria even without functionalization. An important consideration in drug delivery using metallic nanoparticle is toxicity. Tests should be included in early stages of nanoparticle development in appropriate conditions (buffer, pH, temperature) to ensure higher rates of success in in vivo models with no toxicity. Similarly, platinum nanoparticles also exhibit unique antioxidant properties [84] but safety of the particles has not been confirmed [85] in cell free and cell based assays. Platinum nanoparticles are of approximately 20–30 nm and of varying shapes (nanoflowers, spheres, and multipods). The studies confirm that platinum nanoparticles of various shapes do not exhibit cytotoxicity as they do not produce ROS in a cell free system or inflammatory responses (interleukin-6, and tumor necrosis factor-α) in a human umbilical vein endothelial cell (HUVEC) system up to 50 μg of platinum nanoparticles. They have been known for their antioxidant properties and potential mitochondria therapeutic effects. Hikosaka et al. demonstrated that platinum particles functionalized with pectin were capable of oxidizing NADH to NAD^+ [86]. This property may be used to normalize the redox potential by regenerating NAD^+ species for glycolysis and other cellular pathways to function properly. It is also demonstrated that platinum nanoparticles (with a size of 5 nm) are capable of quenching superoxide anion radical (O^{2-}) and hydrogen peroxide (H_2O_2) [87]. They may mimic the functions of complex I since pectin functionalized platinum nanoparticles oxidize NADH to NAD^+ and reduce ubiquinone

(CoQ) to ubiquinol (CoQH2). These nanoparticles have unique therapeutic benefits in diseases with complex I deficiencies such as Alzheimer's disease. Moreover, polyacrylic acid (PAA) protected platinum nanoparticles of approximately 2 nm are also capable of acting as antioxidants in vitro.

The polyacrylic acid (PAA) coated platinum nanoparticles scavenge the superoxide anion radicals in a dose dependent manner. Since PAA alone was not capable of scavenging the radicals, the platinum could be responsible for the antioxidant activity. In a pulmonary inflammation model, antioxidant properties were demonstrated by platinum nanoparticles in vitro as well as in vivo. PAA coated platinum nanoparticles can effectively scavenge ROS and may be more effective than available current antioxidant treatments. It has been reported that zinc oxide (ZnO) nanoparticles lead to the alteration of the electron transport chain, triggering the apoptotic pathways of the cell and induce mitochondrial cytotoxicity in human colon carcinoma LoVo cells [88].

7.9 Conclusion

Mitochondria play a crucial role in various metabolic processes of the cell and mitochondrial dysfunction is related to several diseases. The mitochondrion is a novel intracellular target involved in the pathology of many degenerative and metabolic diseases. Although mitochondria appeared to be a potential target for many diseases, the knowledge about functional characteristics in relation to diseases and drug delivery into the mitochondria is still in its infancy. This is certainly due to the barriers that have to be circumvented to achieve a selective targeting and accumulation in mitochondria. Even though several approaches to mitochondrial drug delivery have already been accomplished, there is still a demand for more selective targeting strategies with improved drug efficiency and the therapeutic outcome in various diseases. Currently, the relationships between mitochondrial dysfunction and related disease are being investigated and established. Pharmaceutical nanocarriers offer great promise for site specific mitochondrion delivery. However, the development of these multifunctional nanoparticles for clinical applications has proven to be challenging and they are still at an early stage.

References

1. Ferreira L, Karp JM, Nobre L, Langer R (2008) New opportunities: the use of nanotechnologies to manipulate and track stem cells. Cell Stem Cell 3:136–146
2. Durazo SA, Kompella UB (2012) Functionalized nanosystems for targeted mitochondrial delivery. Mitochondrion 12:190–201
3. D'Souza GGM, Weissig V (2009) Subcellular targeting: a new frontier for drug-loaded pharmaceutical nanocarriers and the concept of the magic bullet. Expert Opin Drug Deliv 6:1135–1148

4. Murphy MP, Smith RAJ (2000) Drug delivery to mitochondria: the key to mitochondrial medicine. Adv Drug Deliv Rev 41:235–250
5. Szewczyk A, Wojtczak L (2002) Mitochondria as a pharmacological target. Pharmacol Rev 54:101–127
6. Detmer SA, Chan DC (2007) Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol 8:870–879
7. Wallace KB, Starkov AA (2000) Mitochondrial targets of drug toxicity. Annu Rev Pharmacol Toxicol 40:353–388
8. DiMauro S (2004) Mitochondrial diseases. Biochim Biophys Acta 1658:80–88
9. Lowell BB, Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. Science 307:384–387
10. Gibson GE, Karuppagounder SS, Shi Q (2008) Oxidant-induced changes in mitochondria and calcium dynamics in the pathophysiology of Alzheimer's disease. Mitochon Oxid Stress Neurodegen Disord. Ann N Y Acad Sci 1147:221–232
11. Pathania D, Millard M, Neamati N (2009) Opportunities in discovery and delivery of anticancer drugs targeting mitochondria and cancer cell metabolism. Adv Drug Deliv Rev 61: 1250–1275
12. Yamada Y, Shinohara Y, Kakudo T, Chaki S, Futaki S, Kamiya H et al (2005) Mitochondrial delivery of mastoparan with transferring liposomes equipped with a pH-sensitive fusogenic peptide for selective cancer therapy. Int J Pharm 303:1–7
13. Muratovska A, Lightowlers RN, Taylor RW, Wilce JA, Murphy MP (2001) Targeting large molecules to mitochondria. Adv Drug Deliv Rev 49:189–198
14. Horobin RW, Trapp S, Weissig V (2007) Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria. J Control Release 121:125–136
15. Langner M (2000) The intracellular fate of non-viral DNA carriers. Cell Mol Biol Lett 5:295–313
16. Bulmus V (2005) Biomembrane-active molecular switches as tools for intracellular drug delivery. Aust J Chem 58:411–422
17. Gupta B, Levchenko TS, Torchilin VP (2005) Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev 57:637–651
18. Torchilin VP (2006) Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. Annu Rev Biomed Eng 8:343–375
19. Luby-Phelps K (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int Rev Cytol 192:189–221
20. Ellis RJ, Minton AP (2003) Cell biology: join the crowd. Nature 425:27–28
21. Seksek O, Biwersi J, Verkman AS (1997) Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. J Cell Biol 138:131–142
22. Salnikov V, Lukyanenko YO, Frederick CA, Lederer WJ, Lukyanenko V (2007) Probing the outer mitochondrial membrane in cardiac mitochondria with nano-particles. Biophys J 92:1058–1071
23. Crompton M, Virji S, Ward JM (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur J Biochem 258:729–735
24. Jensen RE, Dunn CD (2002) Protein import into and across the mitochondrial inner membrane: role of the TIM23 and TIM22 translocons. Biochim Biophys Acta 1592:25–34
25. Paunesku T, Vogt S, Lai B, Maser J, Stojicevic N, Thurn KT et al (2007) Intracellular distribution of TiO_2-DNA oligonucleotide nanoconjugates directed to nucleolus and mitochondria indicates sequence specificity. Nano Lett 7:596–601
26. Biswas S, Dodwadkar NS, Piroyan A, Torchilin VP (2012) Surface conjugation of triphenylphosphonium to target poly(amidoamine) dendrimers to mitochondria. Biomaterials 33:4773–4782
27. Smith RAJ, Murphy MP (2011) Mitochondria-targeted antioxidants as therapies. Discov Med 11:106–114

28. Liberman E, Skulachev V (1970) Conversion of biomembrane-produced energy into electric form. IV. Biochim Biophys Acta 216:30–42
29. Jauslin ML, Meier T, Smith RA, Murphy MP (2003) Mitochondria-targeted antioxidants protect Friedreich Ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants. FASEB J 17:1972–1974
30. Sheu SS, Nauduri D, Anders MW (2006) Targeting antioxidants to mitochondria: a new therapeutic direction. Biochim Biophys Acta 1762:256–265
31. Fernandez-Carneado J, VanGool M, Martos V, Castel S, Prados P, de Mendoza J, Giralt E (2005) Highly efficient, nonpeptidic oligoguanidinium vectors that selectively internalize into mitochondria. J Am Chem Soc 127:869–874
32. Yousif LF, Stewart KM, Horton KL, Kelley SO (2009) Mitochondria-penetrating peptides: sequence effects and model cargo transport. Chembiochem 10:2081–2088
33. Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW et al (2004) Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol Chem 279:34682–34690
34. Szeto HH (2006) Mitochondria-targeted peptide antioxidants: novel neuroprotective agents. AAPS J 8:E277–E283
35. Horton KL, Stewart KM, Fonseca SB, Guo Q, Kelley SO (2008) Mitochondria-penetrating peptides. Chem Biol 15:375–382
36. Sebbage V (2009) Cell-penetrating peptides and their therapeutic applications. Bioscience Horizons 2:64–72
37. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ et al (2002) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem 278:585–590
38. Pfanner N, Geissler A (2001) Versatility of the mitochondrial protein import machinery. Nat Rev Mol Cell Biol 2:339–349
39. Schatz G (1996) The protein import system of mitochondria. J Biol Chem 271:31763–31766
40. Zhang C, Sriratana A, Minamikawa T, Nagley P (1998) Photosensitisation properties of mitochondrially localised green fluorescent protein. Biochem Biophys Res Commun 242:390–395
41. Weissig V (2003) Mitochondrial-targeted drug and DNA delivery. Crit Rev Ther Drug Carrier Syst 20:1–62
42. Sneh-Edri H, Likhtenshtein D, Stepensky D (2011) Intracellular targeting of PLGA nanoparticles encapsulating antigenic peptide to the endoplasmic reticulum of dendritic cells and its effect on antigen cross-presentation in vitro. Mol Pharm 8:1266–1275
43. Zhou J, Zhao W-Y, Ma X, Ju R-J, Li X-Y, Li N, Sun M-G, Shi J-F, Zhang C-X, Lu W-L (2013) The anticancer efficacy of paclitaxel liposomes modified with mitochondrial targeting conjugate in resistant lung cancer. Biomaterials 34:3626–3638
44. Li N, Zhang CX, Wang XX, Zhang L, Ma X, Zhou J, Ju RJ, Li XY, Zhao WY, Lu WL (2013) Development of targeting lonidamine liposomes that circumvent drug-resistant cancer by acting on mitochondrial signaling pathways. Biomaterials 34:3366–3380
45. Yu Y, Wang Z-H, Zhang L, Yao H-J, Zhang Y, Li R-J, Ju R-J, Wang X-X, Zhou J, Li N, Lu W-L (2012) Mitochondrial targeting topotecan-loaded liposomes for treating drug-resistant breast cancer and inhibiting invasive metastases of melanoma. Biomaterials 33:1808–1820
46. Malhi SS, Budhiraja A, Arora S, Chaudhari KR, Nepali K, Kumar R, Sohi H, Murthy RS (2012) Intracellular delivery of redox cycler-doxorubicin to the mitochondria of cancer cell by folate receptor targeted mitocancerotropic liposomes. Int J Pharm 432:63–74
47. Biswas S, Dodwadkar NS, Deshpande PP, Torchilin VP (2012) Liposomes loaded with paclitaxel and modified with novel triphenylphosphonium-PEG-PE conjugate possess low toxicity, target mitochondria and demonstrate enhanced antitumor effects in vitro and in vivo. J Control Release 159:393–402
48. Solomon MA, Shah AA, D'Souza GG (2013) In Vitro assessment of the utility of stearyl triphenyl phosphonium modified liposomes in overcoming the resistance of ovarian carcinoma Ovcar-3 cells to paclitaxel. Mitochondrion 134:64–72
49. Wang L, Liu Y, Li W, Jiang X, Ji Y, Wu X et al (2011) Selective targeting of gold nanorods at the mitochondria of cancer cells: implications for cancer therapy. Nano Lett 11:772–780

50. Wang X-X, Li Y-B, Yao H-J, Ju R-J, Zhang Y, Li R-J, Yu Y, Zhang L, Lu W-L (2011) The use of mitochondrial targeting resveratrol liposomes modified with a dequalinium polyethylene glycol-distearoylphosphatidyl ethanolamine conjugate to induce apoptosis in resistant lung cancer cells. Biomaterials 32:5673–5687
51. Patel NR, Hatziantoniou S, Georgopoulos A, Demetzos C, Torchilin VP, Weissig V et al (2010) Mitochondria-targeted liposomes improve the apoptotic and cytotoxic action of sclareol. J Liposome Res 20:244–249
52. Liguori L, Marques B, Villegas-Mendez A, Rothe R, Lenormand J-L (2008) Liposomes-mediated delivery of pro-apoptotic therapeutic membrane proteins. J Control Release 126:217–227
53. Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y, Yamashita K, Kobayashi H, Kikuchi H, Harashima H (2008) MITO-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. Biochim Biophys Acta 1778:423–432
54. Boddapati SV, D'Souza GG, Erdogan S, Torchilin VP, Weissig V (2008) Organelle-targeted nanocarriers: specific delivery of liposomal ceramide to mitochondria enhances its cytotoxicity in vitro and in vivo. Nano Lett 8:2559–2563
55. Hoshino K, Fujioka T, Oku S, Nakamura M, Suga Y, Yamaguchi K et al (2004) Quantum dots targeted to the assigned organelle in living cells. Microbiol Immunol 48:985–994
56. Foley S, Crowley C, Smaihi M, Bonfils C, Erlanger BF, Seta P et al (2002) Cellular localization of a water-soluble fullerene derivative. Biochem Biophys Res Commun 294:116–119
57. Cuchelkar V, Kopeckova P, Kopecek J (2008) Novel HPMA copolymer-bound constructs for combined tumor and mitochondrial targeting. Mol Pharm 5:776–786
58. García-Péreza AI, Galeanob E, Nietoa E, Sancho P (2011) Dequalinium induces human leukemia cell death by affecting the redox balance. Leuk Res 35:1395–1401
59. D'Souza GG, Cheng SM, Boddapati SV, Horobin RW, Weissig V (2008) Nanocarrier-assisted sub-cellular targeting to the site of mitochondria improves the pro-apoptotic activity of paclitaxel. J Drug Target 16:578–585
60. D'Souza GG, Rammohan R, Cheng SM, Torchilin VP, Weissig V (2003) DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells. J Control Release 92:1–2
61. Gao X, Kim KS, Liu D (2007) Nonviral gene delivery: what we know and what is next. AAPS J 9:E92–E104
62. Vaidya B, Paliwal R, Rai S, Khatri K, Goyal AK, Mishra N, Vyas SP (2009) Cell-selective mitochondrial targeting: a new approach for cancer therapy. Cancer Ther 7:141–148
63. Inoki Y (2000) Proteoliposomes colocalized with endogenous mitochondria in mouse fertilized egg. Biochem Biophys Res Commun 278:183–191
64. Miller BR, Cumsky MG (1991) An unusual mitochondrial import pathway for the precursor to yeast cytochromec oxidase subunit Va. J Cell Biol 112:833–841
65. Herrmann JM, Koll H, Cook RA, Neupert W, Stuart RA (1995) Topogenesis of cytochrome oxidase subunit II. Mechanisms of protein export from themitochondrial matrix. J Biol Chem 270:27079–27086
66. Rajendran L, Knölker HJ, Simons K (2010) Subcellular targeting strategies for drug design and delivery. Nat Rev Drug Discov 9:29–42
67. Stover TC, Sharma A, Robertson GP, Kester M (2005) Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma. Clin Cancer Res 11:3465–3474
68. Yamada Y, Furukawa R, Yasuzaki Y, Harashima H (2011) Dual function MITO-Porter, a nano carrier integrating both efficient cytoplasmic delivery and mitochondrial macromolecule delivery. Mol Ther 19:1449–1456
69. Yamada Y (2007) Mitochondrial drug delivery and mitochondrial disease therapy – an approach to liposome-based delivery targeted to mitochondria. Mitochondrion 7:63–71
70. Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. Biochem J 435:297–312
71. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M et al (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci 84:7413–7417

72. Reddy MK, Wu L, Kou W, Ghorpade A, Labhasetwar V (2008) Superoxide dismutase-loaded PLGA nanoparticles protect cultured human neurons under oxidative stress. Appl Biochem Biotechnol 151:565–577
73. Marrache S, Tundup S, Harn DA, Dhar S (2013) Ex vivo programming of dendritic cells by mitochondria-targeted nanoparticles to produce interferon-gamma for cancer immunotherapy. ACS Nano 7(8):7392–7402
74. Nishiyama N (2009) Enhanced photodynamic cancer treatment by supramolecular nanocarriers charged with dendrimer phthalocyanine. J Control Release 133:245–251
75. Link S, El-Sayed MA (1999) Size and temperature dependence of the plasmon absorp-tion of colloidal gold nanoparticles. J Phys Chem B 103:4212–4217
76. El-Sayed IH, Huang X, El-Sayed MA (2005) Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer. Nano Lett 5:829–834
77. Flanagan MT, Pantell RH (1984) Surface plasmon resonance and immunosensors. Electron Lett 20:968–970
78. Goodman CM, McCusker CD, Yilmaz T, Rotello VM (2004) Toxicity of gold nano-particles functionalized with cationic and anionic side chains. Bioconjug Chem 15:897–900
79. Esumi K, Takei N, Yoshimura T (2003) Antioxidant-potentiality of gold-chitosan nanocomposites. Colloids Surf B Biointerfaces 32:117–123
80. Qi LF, Xu ZR, Li Y, Jiang X, Han XY (2005) In vitro effects of chitosan nanoparticles on proliferation of human gastric carcinoma cell line MGC803 cells. World J Gastroenterol 11:5136–5141
81. Esumi K, Houdatsu H, Yoshimura T (2004) Antioxidant action by gold-PAMAM den-drimer nanocomposites. Langmuir 20:2536–2538
82. Isomaa B, Reuter J, Djupsund BM (1976) The subacute and chronic toxicity of cetyltrimethylammonium bromide (CTAB), a cationic surfactant, in the rat. Arch Toxicol 35:91–96
83. Suzuki H, Toyooka T, Ibuki Y (2007) Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis. Environ Sci Technol 41:3018–3024
84. Aiuchi T, Nakajo S, Nakaya K (2004) Reducing activity of colloidal platinum nanopar-ticles for hydrogen peroxide, 2,2-diphenyl-1-picrylhydrazyl radical and 2,6-dichlorophenol indophenol. Biol Pharm Bull 27:736–738
85. Elder A, Yang H, Gwiazda R, Teng X, Thurston S, He H et al (2007) Testing nanomaterials of unknown toxicity: an example based on platinum nanoparticles of different shapes. Adv Mater 19:3124–3129
86. Hikosaka K, Kim J, Kajita M, Kanayama A, Miyamoto Y (2008) Platinum nanoparticles have an activity similar to mitochondrial NADH: ubiquinone oxidoreductase. Colloids Surf B Biointerfaces 66:195–200
87. Kajita M, Hikosaka K, Iitsuka M, Kanayama A, Toshima N, Miyamoto Y (2007) Platinum nanoparticle is a useful scavenger of superoxide anion and hydrogen peroxide. Free Radic Res 41:615–626
88. De Berardis B, Civitelli G, Condello M, Lista P, Pozzi R, Arancia G et al (2010) Exposure to ZnO nanoparticles induces oxidative stress and cytotoxicity in human colon carcinoma cells. Toxicol Appl Pharmacol 246:116–127

Chapter 8
Targeted Delivery of Nucleic Acid Therapeutics via Nonviral Vectors

Mamta Kapoor and Diane J. Burgess

8.1 Introduction

Nucleic acid (NA) therapeutics are deoxyribonucleic or ribonucleic acid (DNA/RNA) based molecules that regulate expression of a particular gene, intracellularly. In the early stages of development, NA based therapy was employed to correct inheritable disorders resulting from a single gene defect such as cystic fibrosis [1] and severe combined immunodeficiency disease (SCID) [2, 3]. Later, this therapy was also useful for complex genetic disorders such as cancer [4, 5], and cardiovascular [6, 7] and neurodegenerative diseases [8, 9]. The first gene therapy clinical trial was in 1990, for treatment of adenosine deaminase (ADA) deficiency [10], a genetic disorder that weakens the immune system to fight against infections. Normal gene encoding for ADA was inserted into the patient's white blood cells and resulted in normal production of ADA. This outcome together with the successful mapping of the human genome in the year 2003 [11], encouraged many scientists to work in this area and has led to many gene based clinical trials [12] as well as commercial gene products [13, 14].

Despite these promising developments, the full potential of gene therapy remains to be exploited. One of the key challenges to successful NA therapeutics is their inefficient delivery to the desired site. This is a result of their susceptibility to enzymatic degradation, poor cellular uptake and poor endosomal escape. For DNA-based therapeutics (such as plasmid DNA), entry into the nucleus is an additional challenge. Currently, numerous strategies are being pursued to improve delivery of

M. Kapoor
Department of Pharmaceutics, University of Minnesota,
Minneapolis, MN, USA

D.J. Burgess (✉)
School of Pharmacy, University of Connecticut, Storrs, CT, USA
e-mail: Diane.burgess@uconn.edu

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_8

NAs in vitro as well as in vivo. Although high levels of DNA transfection have been achieved by mechanical methods such as microinjection [15, 16] particle bombardment [17] and electroporation [18], these methods may cause local toxicity and are impractical for in vivo purposes. Alternate methods include chemical modification of NA structure in the region of the phosphate backbone, sugars, or bases [19–22]; however, this may compromise NA bioactivity and stability [22, 23]. Accordingly, the use of viral or nonviral delivery vectors is preferred. Viral vectors have very high transfection efficiency due to their innate components that facilitate efficient cellular penetration through the plasma membrane, the endosomal membrane and the nuclear membrane [24, 25]. Nonetheless, viral vectors may be immunogenic as well as toxic [26, 27]. Consequently, utilization of nonviral vectors is favorable in order to facilitate efficient intracellular transport of NAs.

Nonviral vectors such as cationic liposomes and polymers (including dendrimers) interact electrostatically with NAs, thereby protecting these against enzymatic attack, improving their cellular internalization as well as their endosomal escape. However, a major drawback to the use of these cationic substances is their considerable local or systemic toxicity upon administration [28–30]. Accordingly, focus has been diverted towards anionic liposomes/polymers for efficient gene delivery [31–36]. Other nonviral vectors used for gene delivery include PLGA polymeric nanoparticles, which are useful for sustained delivery of NAs. Most recent among the nonviral vectors are "microbubbles" which release NAs upon exposure to ultrasound [37–41].

For safe and efficient delivery, it is imperative to deliver the therapeutic molecule to the desired site while leaving the healthy cells unaffected. This can be attained by using targeted (or site-specific) gene delivery systems. Targeting can be achieved by using polyethylene glycol (PEG) that prolongs particle blood circulation (*passive targeting*) [42] or by using targeting ligands which bind specifically to receptors that are (over-) expressed in diseased cells (*active targeting*). Using these approaches tissue-specific [43], cell-specific [35, 44–47] and even organelle-specific targeting [24, 48–54] can be obtained. Additionally other strategies such as ultrasound-triggered microbubble delivery and suicide gene delivery can be employed [37–41] to achieve site-specific delivery.

This chapter describes nucleic acid therapeutics in preclinical and clinical studies, various nonviral vectors employed to deliver these therapeutic molecules, and several strategies adopted to achieve in vitro/in vivo targeted gene delivery.

8.2 Nucleic Acid Therapeutics

Various NA therapeutics have been employed to treat genetic disorders either by inhibiting expression of harmful proteins (e.g., *antisense oligonucleotides*, *DNAzymes*, *RNAzymes*, *aptamers*, *silencing RNAs and short hairpin RNAs*) or by providing a (missing) therapeutic protein (e.g., *plasmid DNA*). Certain molecules such as *microRNA* can be used for either of the aforementioned purposes.

Figure 8.4 illustrates the site of action of various NA therapeutic molecules. Several of these molecules are currently in clinical trials (Table 8.1). Properties of such NA therapeutics are discussed in the following sections.

8.2.1 Plasmid DNA (pDNA)

pDNA is a small circular double-stranded DNA that transcribes (to messenger RNA) and translates to produce a "therapeutic" protein. Recombinant pDNA has been utilized to provide "therapeutic" proteins in the body to cure various fatal diseases including cancer. For example Gendicine and Ocorine are pDNA based marketed products (China) that efficiently deliver tumor suppressor gene, p53, for the treatment of head and neck cancer [55]. pDNA can also be employed as vaccines for genetic immunization. These can be prepared by cloning the plasmid with genes encoding for antigen(s) from a specific pathogen. Upon intracellular accumulation in "host" cells, DNA vaccine produces the pathogen's antigen that activates the "host" immune system [56]. There are currently around 89 clinical trials based on DNA vaccine, mostly for the treatment of cancer or human immunodeficiency virus (HIV) infection [57]. Other than vaccines, pDNA is also used in cancer suicide gene therapy (CSGT). There are two approaches for CSGT: (1) pDNA is cloned with a cancer-specific promoter sequence of a suicidal gene so that it is upregulated (expressed) only in cancer cells, thereby killing them. Examples of cancer specific promoters include carcinoembryonic antigen (gastric and colorectal carcinomas), telomerase (many tumors including ovarian cancer), and prostate specific antigen (prostate cancer) [58]. (2) pDNA is cloned with genes encoding for enzymes that participate in activation of anticancer prodrugs. The prodrug is targeted to the "disease" site and upon enzymatic activation a toxic metabolite is produced which causes tumor cell death. For example pDNA encoding for *cytosine deaminase* enzyme has been utilized for treating cancer upon activating 5-fluorocytosine [59–61].

8.2.2 DNAzymes and RNAzymes

DNAzyme (DNA enzyme) is DNA-based enzyme that catalytically cleaves messenger RNA (mRNA), thereby interfering with the translation process. After cleavage, mRNA dissociates with DNAzyme and this prepares DNAzyme to cleave other mRNA molecules [62]. In this manner, DNAzyme can inhibit expression of "harmful" proteins. For example, anti-latent membrane protein-1 (LMP1) DNAzyme has been utilized to effectively knockdown expression of LMP-1 proteins (in mice), which are otherwise responsible for nasopharyngeal carcinoma (NPC) [63]. RNAzyme (RNA enzyme) works in a similar fashion as DNAzyme but is less chemically stable due to the susceptibility of the 2′OH group in the ribose sugar to alkaline hydrolysis [64]. Other examples of DNAzyme/RNAzyme are listed in Table 8.1.

Table 8.1 Gene delivery systems in clinical trials [57]

Nucleic acid therapeutic	Product	Target	Disease	Route	Formulation	Status	Company/sponsor
Plasmid DNA—vaccine	Synchrovax SEM vaccine	MKC1106-MT	Stage IV melanoma	Intranodal	–	Phase II	Mannkind Corporation
Plasmid DNA	Gendicine	p-53 tumor suppression gene	Advanced head and neck cancer	Intratumoral	Adeno-associated virus encoding p53 gene	Chinese-FDA	Shenzhen SiBione GeneTech
Plasmid DNA	DOTAP:Chol-fus1	Fus-1 gene	Non-small-cell lung cancer	Intravenous	Lipoplexes	Phase I	M.D. Anderson Cancer Center
Plasmid DNA	(SGT53-01)	p-53 tumor suppression gene	Advanced solid tumors	Intravenous	Anti-T ransferrin receptor scFv lipoplexes with docetaxel	Phase I	SynerGene Therapeutics, Inc
DNAzyme	DZ1	EBV-LMP1	Nasopharyngeal carcinoma	Intratumoral	–	Phase I/lia	Xiangya Hospital of Central South University
DNAzyme	SB010	GATA-3 mRNA	Mild asthma	Inhalational	–	Phase II	Sterna Biologicals GmbH & Co. KG
Ribozyme	OZ1	HIV-1	Human immunodeficiency virus infection	Ex-vivo (Injected hematopoietic progenitor cells)	–	Phase II	Janssen-Cilag Pty Ltd

Aptamer (PEGylated)	E10030	PDGF	Neovascular age-related macular degeneration	Intravitreal	–	Phase II	Ophthotech Corporation
RNA aptamer	Pegaptanib (Macugen)	VEGF165	Age related macular degeneration	Intravitreal	–	US FDA approved	Lawson Health Research Institute, Pfizer
DNA aptamer	AS1411	Nucleolin, NFkappaB	Acute myeloid leukemia	Intravenous	–	Phase II	Antisoma Research
Antisense oligonucleotides	AVI-4658	Dystrophin exon 51	Duchenne muscular dystrophy	Intramuscular	–	Phase I/IIa	Imperial College London
Antisense oligonucleotides	Miravirsen	miR-122	Hepatitis C virus infection	Intravenous	–	Phase II	Santaris Pharma
Antisense oligonucleotides	Alicaforsen	ICAM-1	Pouchitis	Rectal	–	FDA apporoved	Atlantic Pharmaceuticals Limited
Antisense oligonucleotides	Mipomersen	Apolipoprotein B	Severe LDL-hypercholesterolemia	Subcutaneous	–	Phase III	Ludwig-Maximilians-University of Munich
siRNA	Atu-027	Proteinkinase N3	Advanced solid tumor	Intravenous	Lipoplex	Phase I	Silence Therapeutics
siRNA	ALN-VSP-02	Kinesin spindle protein (KSP) and VEGF	Liver cancer	Intravenous	SNALP (stable nucleic acid-lipid particle)	Phase I	Alnylam Pharmaceuticals

(continued)

Table 8.1 (continued)

Nucleic acid therapeutic	Product	Target	Disease	Route	Formulation	Status	Company/sponsor
siRNA	CALAA-01	M2 subunit of ribonuclease reductase (R2)	Solid tumors	Intravenous	Cyclodextrim containing CAL-101, PEG, PEG-Tf	Phase I	Calando Pharmaceuticals
siRNA	C and 5 (Bevasiranib sodium)	VEGF	Wet Age-related macular disease	Intravitreal	–	Phase III	Opko Health, Inc
siRNA	STMN1-shRNA	Stathmin 1	Advanced or metastatic cancer	Intratumoral	Lipoplex	Phase I	Gradalis, Inc.
siRNA-vaccine	FANG™ autologous tumor cell vaccine	Furin	Advanced Melanoma	Intradermal	–	Phase II	Gradalis, Inc.

8.2.3 Aptamers

Nucleic acid aptamers are short single/double stranded NAs that specifically bind (lock and key mechanism) to proteins, other NAs, or even small molecules. Aptamers can be designed to interfere with functioning of "harmful" proteins, either directly (bind to protein) or indirectly (bind to mRNA/DNA). For example, Pegaptanib (Macugen®) is a USFDA approved anti-angiogenic RNA aptamer that prevents blood vessel growth by directly binding to intracellular $VEGF_{165}$ (isoform of vascular endothelial growth factor (VEGF) that regulates vascular permeability). Pegaptanib is therefore useful in the treatment of wet-age related macular degeneration (wet-AMD) (Table 8.1). ARC1779 is a DNA aptamer in Phase II clinical trials for the treatment of thrombotic microangiopathies and carotid artery disease. ARC1779 specifically interacts with von Willebrand factor (protein that recruits platelets to the damaged arteries) and blocks its binding to platelet membrane glycoprotein receptors, resulting in an antithrombotic effect [65]. Other examples of aptamer-based therapy have been reviewed elsewhere [66, 67].

8.2.4 Antisense Oligonucleotides

These are short double stranded DNA/RNA molecules that complimentarily bind to a specific mRNA, release RNase H enzymes, thereby causing mRNA hydrolysis and interference in the translation process [68]. Accordingly, antisense oligonucleotides have been successfully employed to inhibit production of "disease causing" proteins and cure conditions such as hypercholesterolemia [57], neuromuscular disorders [69] and inflammatory disorders [70]. For example Alicaforsen is an FDA approved antisense oligonucleotide for the treatment of pouchitis [70]. Pouchitis is an inflammation of the surgically constructed internal pouch created in ulcerative colitis patients who have had their diseased colons removed. Alicaforsen prevents the production of intercellular adhesion molecule 1, or ICAM-1 protein overexpressed in pouchitis. Another antisense drug Mipomersen, in Phase III clinical trials, acts against apo-B protein to treat severe LDL-hypercholesterolemia [57]. Other examples are summarized in Table 8.1.

8.2.5 shRNA, miRNA, and siRNA

These are the most recent NA therapeutics employed for gene delivery. Short hairpin RNA or shRNA is an expression vector that post-nuclear entry, transcribes to RNA which binds sequence specifically to mRNA and degrades it. For example STMN1-shRNA lipoplexes (Phase-I) have been shown to inhibit production of stathmin 1 (STMN 1) protein. This protein is known to participate in spindle

dynamics associated with advanced or metastatic cancer [57]. Micro-RNAs or miRNAs are physiological molecules that play a key role in normal functioning of the human body [71]. Structurally these are small noncoding endogenous oligonucleotides that bind nonspecifically to the 3′UTR (untranslated region) of cytosolic messenger RNA and (up/down) regulates gene expression [72]. However, miRNA levels are usually disrupted in disease conditions. Accordingly miRNAs needs to be exogenously delivered to restore this balance. For example, endogenous levels of microRNA "miR-34," a tumor suppressor, is usually low in many types of cancers. Accordingly, synthetic miR-34 has been delivered, either directly or with nanoparticles (NOV-340), in various animal models to effectively cure cancer. This could potentially be the first miRNA therapeutic to enter the clinics [73].

Due to nonspecific binding with mRNA, miRNAs could be effective against 1,000s of mRNA molecules with similar sequences [74] but this also increases the probability for off-target effects [75]. Such effects are less common with silencing RNAs (siRNAs) that sequence specifically bind to the mRNA [76]. siRNAs are small 21–25 base nucleotides that function in the cell cytoplasm [77]. Since siRNA is highly specific, compared to miRNA and does not require nuclear delivery (as is the case for shRNA) it is the most preferred in the category of RNA-based therapeutics. Additionally, siRNA is more potent than other therapies such as antisense oligonucleotides since one siRNA molecule can cleave several mRNA molecules (of the same kind) [78]. Many siRNA products are currently in clinical trials for the treatment of a wide range of indications [79] including solid tumors, liver cancer and wet-AMD (Table 8.1).

8.3 Barriers to Efficient Nucleic Acid Delivery

8.3.1 Enzymatic/Chemical Instability

Owing to their structure, NAs are prone to attack by exonucleases as well as endonucleases present in the extracellular/intracellular environment. Accordingly, these get degraded much before reaching the desired site of action. Several efforts have been made to improve NA stability. These include chemical modification of the phosphate backbone, sugars or nucleotide bases [19–22]. Phosphodiester bonds in the NA backbone have been substituted with enzymatically resistant bonds such as phosphorothionate [19], boranophosphates [20]. phosphoramidate, and methylphosphonate. Of these, phosphorothionates have achieved the greatest success [80] as evident by the fact that one of the FDA approved NA products, is Vitravene, an antisense phosphorothionate DNA oligonucleotide. Intravitreal injection of Vitravene is used for the treatment of cytomegalovirus retinitis [81, 82]. Whereas such backbone modifications have improved NA stability, high degree of modification may compromise the safety aspect of NAs. For example Amarzguioui et al. reported that 100 % replacement of phosphodiester bonds with phosphorothioate

caused significant increase in cytotoxicity although there was remarkable improvement in siRNA serum stability [23].

With respect to sugar modification, the ribose sugar in RNA, has been modified with groups such as 2′NH_2, 2′F, 2′OMe, or 2′deoxy [21, 83]. These modifications improved chemical stability of RNA which is otherwise prone to alkaline hydrolysis owing to the presence of 2′OH group [64]. Some researchers have also attempted modification of bases in nucleotides, but this lowered the activity of biomolecules [22].

8.3.2 Immunogenicity

Nucleic acid therapeutics such as siRNAs could be immunogenic since these trigger activation of inflammatory toll-like receptors (TLR) that further activate interferon (IFN-α) [84], interleukins (IL-6) and other cytokines associated with innate immunity [85], Ribose sugar and multiple uridine bases in siRNA have been observed to be responsible for this triggering mechanism, as these are predominantly identified by TLR7 [86]. Accordingly, modification of the siRNA structure can reduce immunogenicity. For example, when a few 2′ OH groups in ribose sugar were substituted with 2′OMe, siRNA immunogenicity was significantly reduced [87, 88]. The precise mechanism, later discovered by Robbins and co-workers, revealed that 2′OMe acts as an antagonist of TLR7, the primary receptor involved in immune stimulation [89]. These findings were also supported by the work of Cekaite et al. [90]. Besides 2′OMe, other groups such as 2′F [90] and locked NA (LNA) [84] are also useful to inhibit the immune response. In addition to TLR, other cellular receptors such as PKR (dsRNA-binding protein kinase) [91] and RIG-1 (retinoic acid-inducible gene-I) [92] have been implicated in immune-stimulation by siRNA.

8.3.3 Nuclear Entry

Other than cellular uptake and endosomal release (Sect. 3.4), DNA based therapeutics (pDNA, shRNA) have the additional challenge of entering into the nucleus where transcription occurs (Fig. 8.4). Various strategies have been adopted to improve nuclear entry of these biomolecules and these are discussed in Sect. 5.2.2.1.

8.3.4 Poor Cellular Uptake and Endosomal Escape

Attributed to their relatively large molecular weight compared to small molecules and the presence of a highly negatively charged backbone, NAs face difficulty in passing through the anionic plasma membrane. Additionally, NAs are unable to

escape from endosomes due to the inability of the anionic backbone of NAs to interact with anionic endosomal lipids, leading to their degradation in this low pH environment (Fig. 8.4). The degradation mechanism includes hydrolysis of phosphodiester bonds [93] and/or alterations in base pairing in the acidic pH [94]. To improve cellular uptake as well as the endosomal release capability of NAs, two approaches have been adopted: (a) direct conjugation and (b) delivery vectors. The direct conjugation approach involves chemical conjugation of NAs with moieties (lipids [95], polymers [96], peptides [97]) that can potentiate the cellular uptake and/or endosomal escape of the biomolecule. However, incorrect site of conjugation causing reduced bioactivity [98], charge neutralization of the cationic conjugated moiety by anionic NA [99], and the inability of the conjugated species to protect the NA against enzymatic attack, often limits the potential of this approach. For these reasons, use of delivery vectors is more common.

8.4 Delivery Vectors

Efficient gene delivery for therapeutic approaches is a demanding task that urges the development of delivery vectors capable of overcoming the aforementioned challenges to efficient NA delivery. Delivery vectors not only protect NAs from enzymatic digestion but also enhance their cellular uptake and endosomal release capability. On the basis of their origin and mechanism of action, delivery vectors can be categorized as viral and nonviral.

8.4.1 Viral Vectors

Inherent properties of viruses such as effective membrane penetration (plasma, endosome, or nuclear) has been exploited for achieve very high transfection efficiency with viral vectors [24, 25]. However, some viruses (retrovirus) integrate with host genome and excessively produce viral proteins causing a fatal immune response. For example, retrovirus-based delivery of tumor suppressor gene in an X-lined SCID patient caused massive immune response triggered by the viral vector (viral proteins), leading to multiple organ failure and brain death [100]. For safer delivery, researchers have prepared attenuated viruses lacking in virulent components. Currently there are seven attenuated (adeno-associated) viral products in early phase, and two in late phase (III) clinical trials [57]. Despite this success, future prospects of viral vectors are restricted due to the limit size available for cloning therapeutic genes, scale-up as well as manufacturability issues [101]. Consequently, focus is more towards developing safe and efficient nonviral vector based gene delivery systems.

8.4.2 Nonviral Vectors

Nonviral vectors offer a high level of control with respect to their physicochemical properties and manufacturing on industrial scale. Additionally these are non-immunogenic and relatively safer compared to the viral vectors. However, these vectors have low transfection efficiencies due to lack of innate characteristics of a virus. Tremendous efforts have been made to develop and optimize nonviral carriers which are safe and have transfection efficiency of viral vectors. Various nonviral vectors useful for gene delivery have been discussed in the following sections.

8.4.2.1 Liposomes

Liposomes are spherical structures with an aqueous core surrounded by a lipid bilayer. These are by far the most commonly used nonviral vectors to facilitate NA delivery. Lipids associate with NAs either via surface complexation or encapsulation of hydrophilic NA molecules within the aqueous core. Biophysical properties of liposomes can be modulated to achieve high NA entrapment, efficient cellular uptake and endosomal escape [32]. Due to this flexibility in designing the liposomal formulations, there are several liposome associated NA products in preclinical [79] and clinical studies (see Table 8.1). Both cationic as well as anionic liposomes have been used for delivery of NAs. Structures of commonly used lipids are shown in Fig. 8.1.

Cationic Liposomes

Cationic liposomes electrostatically interact with NAs to form the lipid-NA complexes also known as "lipoplexes." Liposomes help in NA internalization into the cells, as well as its release from endosomes upon interaction with anionic endosomal lipids. The first cationic lipid, DOTMA (Fig. 8.1) was synthesized by Felgner and co-workers [102]. This was followed by other cationic lipids such as DOTAP [103, 104], DOSPA [105], DOSPER [106] and Oligofectamine [44]. Liposomes prepared with these lipids in association with fusogenic lipids (such as di-oleyl phosphatidylethanolamine or DOPE) and/or cholesterol have shown transfection efficiencies in vitro [107–112].

The aforementioned first generation cationic lipids are cytotoxic which restricts their use in vivo [30]. This toxicity could be attributed to their positive charge responsible for nonspecific interactions causing interference with the activity of ion channels, reduction in cellular adhesion and membrane destabilization [113, 114]. Additionally, cationic substances have shown to cause cellular stress in terms of altered actin cytoskeleton and abnormal cell proliferation [115, 116]. Kedmi et al. observed hepatotoxicity and significant weight loss in mice injected with cationic nanoparticles [29]. Ikebe et al. reported triggered release of pro-inflammatory

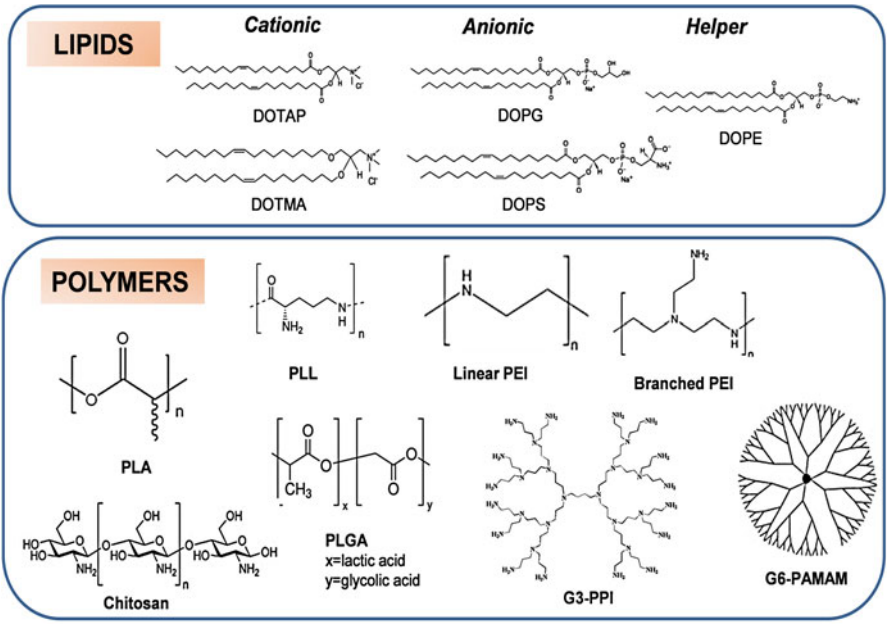

Fig. 8.1 Structures of commonly used nonviral gene delivery vectors. *DOTMA* 1,2-di-O-octadecenyl-3-trimethylammonium propane, *DOTAP* 1,2-dioleoyl-3-trimethylammonium propane, *DOPG*1,2-dioleoyl-sn-glycero-3-phospho-1′-rac-glycerol,*DOPS*1,2-dioleoyl-sn-glycero-3-phospho-L-serine, *DOPE* 1,2-dioleoyl-sn-glycero-3- phosphoethanolamine, *PEI* polyethyleneimine, *PLGA* poly-lactic-co-glycolide, *PLA* polylactic acid, *PPI* polypropyleneimine, *PAMAM* polyamidoamine

cytokines (such as, IL-2, TNF-α) possibly causing increased risk of angiogenesis and tumor progression [28]. Cationic liposomes also interact with negatively charged cellular components (such as serum proteins, opsonins, and enzymes), consequently leading to serum inactivation [117–119].

To overcome the toxicity and serum inactivation issues, efforts have been made to develop second generation cationic lipids with modified structures. The main components of a cationic lipid which are known to regulate transfection efficiency and cytotoxicity are hydrophobic lipid anchor, the linker, and hydrophilic head group. The lipid anchor is typically either a fatty chain (e.g., derived from oleic or myristic acid) or a cholesterol group, which determines the physical properties of the lipid bilayer, such as flexibility, interaction with membrane lipids, and the rate of lipid exchange [109], and these could indirectly impact NA activity. For example lipid tail unsaturation [120–123], shorter chain length [109, 124–126], and double-tailed lipids [127, 128] have been shown to improve the transfection efficiency. The linker group is an important determinant of the chemical stability, biodegradability, and transfection efficiency of the cationic lipid. Biodegradable lipids are being developed that can be metabolized by various enzymes (e.g., esterases, peptidases)

to minimize any toxicity [123, 129]. For example ester linkages are less toxic due to their biodegradability when compared to nonbiodegradable linkers such as ether and carbamate [130–132]. The linker can also provide sites for the introduction of novel side chains to enhance targeting, uptake, and trafficking. The positively charged headgroup on the cationic lipid is responsible for interacting with the negatively charged DNA and is a critical determinant of the transfection and cytotoxicity of liposome formulations. The headgroups differ markedly in structure and may be single- or multiple-charged as primary, secondary, tertiary, and/or quaternary amines. Multivalent headgroups, such as spermine, in a T-shape configuration tend to be more effective than their monovalent counterparts at facilitating gene transfer [109, 133]. Also, the presence of hydroxyalkyl [109], or cyclic groups [134], increases the transfection efficiency. Continued progress toward a comprehensive relationship between lipid structure, its complexation with NA molecules, and the subsequent interaction with the biological system is necessary to facilitate the design of cationic lipids with optimal properties. Such a careful designing of cationic lipids has led to development of commercially available transfection reagents Lipofectamine 2000 (Invitrogen) and RNAiMax (Invitrogen) that are efficient and relatively safer compared to conventional lipid based reagents such as Lipofectin and Lipofectamine [135, 136].

Anionic Liposomes

Owing to the cationic charge based toxicity of first generation cationic lipids, significant work has been done to exploit the potential of anionic liposomes for safe and efficient NA delivery [31, 45, 137, 138]. However, entrapment of NAs within these lipids is challenging due to electrostatic repulsion between the two anionic charged species (lipid and NAs). For example, Foged et al. attempted preparing siRNA associated anionic liposomes. These formulations showed only 7–9 % encapsulation efficiency with no activity in HeLa cells [137]. Accordingly, for efficient lipoplex formation, a third moiety is required which can act as a bridging agent between anionic lipids and NAs. For example, Huang's group prepared anionic lipoplexes using the cationic polymer, poly-L-lysine [35]. Others have prepared anionic liposomal formulations (1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/1,2-dioleoyl-*sn*-glycero-3-phospho-1′-*rac*-glycerol (DOPG), to efficiently deliver antisense oligonucleotides into hippocampal neurons [139] or bacterial cells [140]. Our group has developed anionic lipoplexes wherein anionic liposomes (DOPG/DOPE) have been complexed with DNA/siRNA with calcium ion bridges. These systems have shown equivalent transfection efficiency and no cytotoxicity when compared to commercial cationic liposomes, Lipofectamine™ 2000 in CHO-K1 (DNA lipoplexes) [31, 34, 141] and MDA-MB-231 cells (siRNA lipoplexes) [33]. Figure 8.2 represents confocal images that shows efficient (time-dependent) cellular uptake and endosomal release of anionic lipoplexes in MDA-MB-231 cells.

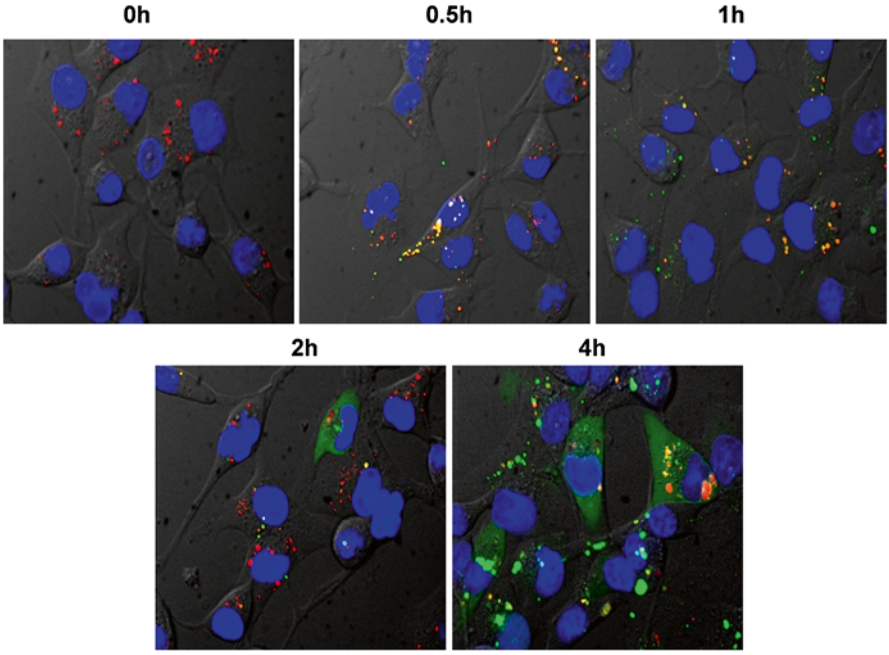

Fig. 8.2 Confocal images of time-dependent (4 h) uptake and endosomal release of siRNA from calcium associated anionic lipoplexes. Cells were stained with Hoescht 33342 (*blue*) for nucleus and LysoTracker red DND-99 for lysosomes. Overlay images of *blue*, *green*, *red*, and transmitted light channels are shown. Reproduced from ref. [33]

8.4.2.2 Polymers

Polymers are macromolecules composed of linear or branched chains of repeated units of smaller molecules (monomers). Several cationic polymers have been employed for efficient gene delivery [142] (Fig. 8.1). Cationic polymers often contain high densities of primary, secondary, tertiary or quaternary amines, which are protonatable at neutral pH [143]. Due to the high density of positive charges cationic polymers self-assemble with DNA/RNA to generate condensed structures (polyplexes) that are usually taken up by cells via endocytosis [143]. Polymers facilitate endosomal release of NAs via the "*proton sponge*" effect [144]. Accordingly, low endosomal pH causes protonation of several amines in the polymer chain resulting in pH buffering. The buffering protons in endosomes attract counter ions (chloride ions) causing osmotic swelling, endosomal rupture, thereby releasing the entrapped NAs.

One of the early polymers used for gene delivery is *Poly*-l-*lysine* (PLL). PLL has low efficiency mainly because of its poor endosomal escaping capability [145] and therefore, it is often co-administered with chloroquine or fusogenic peptides [146, 147]. Alternately, endosomal release can be promoted by conjugating PLL with imidazole containing group such as histidine that gets protonated in acidic pH [148, 149].

Another polymer widely used as efficient gene delivery vector is p*olyethyleneimine* (PEI). PEI has a high charge density due to the presence of nitrogen at every third position in the polymer chain [150] and therefore promotes cellular uptake and endosomal release. For example, linear-PEI (Jet-PEI, 22 kDa) has been utilized to efficiently deliver shRNA-pDNA upon intravitreal injection in mice [151]. For other examples see Table 8.2.

Multiple cationic charges on PEI facilitate effective condensation of NAs as well as promote cellular uptake; however, this also increases PEI cytotoxicity. Therefore, low molecular weight PEIs are favored over higher molecular weight PEIs [152, 153]. PEI toxicity is also contributed by the nonbiodegradable nature of these polymers. Recently, biodegradable PEIs with cleavable linkers such as disulfide [154] and amino esters [155, 156] have been prepared that showed low cytotoxicity and high transfection efficiency when compared to the unmodified PEIs. PEIs high charge density results in multi-contact points with the NA, thereby slowing DNA/RNA dissociation from the polymers, after endosomal escape. This issue was combated by employing linear PEIs instead of branched ones. For example Kleemann et al. showed high efficiency and low toxicity with linear PEI/DNA complexes injected into mice trachea, compared to branched PEI/DNA complexes [157].

Due to the nonbiodegradable nature of conventional PEIs, other biocompatible and biodegradable polymers have been exploited for efficient gene delivery. An example includes *chitosan* which is a linear cationic polymer consisting of glucosamine and N-acetyl D-glucosamine units. Chitosan has shown higher transfection efficiency compared to naked DNA, when injected intratracheally in mice [158]. However, chitosan efficiency was lower than that of PEI (25 kDa) probably due to less effective endosome escaping capability of chitosan. This issue was combated by optimizing degree of deacetylation in chitosan [159]. For example (84 % deacetylated) chitosan/siRNA nanoparticles resulted in efficient knockdown (~44 %) of TNF-alpha gene (inflammation marker) in mouse macrophages and thus were useful in arthritis therapy [160]. Chitosan grafted into PEI has shown to be an efficient (better than PEI alone) and safe gene delivery vector both in vitro as well as in vivo [161].

8.4.2.3 Dendrimers

Dendrimers are structured polymers that have attained recognition as nano-scale units or carriers for a wide range of applications [162]. These carriers are attractive candidates for gene delivery owing to their small condensed size, ease of preparation and functionalization, and their ability to display multiple copies of surface groups for biological recognition processes. Structurally dendrimers comprise a series of branches around an inner core. This unique core-shell design of dendrimers helps in the incorporation of both hydrophilic and hydrophobic moieties [163]. Dendrimers can be synthesized starting from the central core and working out toward the periphery (*divergent synthesis*) or in a top-down approach starting from the outermost residues (*convergent synthesis*) [164].

Table 8.2 Pre-clinical progress of targeted gene delivery systems

Category	Non-viral delivery vector	Nucleic acid therapeutic	Target	Route	In vivo model	References
Cell-surface targeting						
Small molecules						
Folate	Liposomes (RPR209120/DOPE)	pDNA	Luciferase	i.v.	Lung carcinoma	[195]
Asialofutin (asialoglycoproteins)	PLGA (50:50)/DOTAP	pDNA	IL-12	i.t.	Hepatocellular carcinoma	[268]
Galactose	DOT AP/DPPC/Chol/DSPE-PEG galactose	siRNA	Luciferase	i.v.	Hepatitis	[205]
HDL/LDL/cholesterol	SNALP liposomes	siRNA	Apo-B	i.v.	LDL/SR-B1 receptor knockout mice	[277]
Anisamide	PEGylated cyclodextrin	siRNA		i.v.	Prostrate carcinoma	[208]
Peptides						
Enhanced growth factor (EGF)	PEG-PEI	pDNA	Luciferase	i.v.	Hepatocellular carcinoma	[278]
RGD	PEG-PEI	siRNA	VEGF	i.v.	Neuroblastma	[274]
Oligoarginine (9R)	Lipopolymer	siRNA	VEGF	i.t.	Colon ade nocarcinoma xenograft	[269]
Transferrin						
	PEG-PAMAM dendrimers	pDNA	Radiolabelled	i.v.	–	[210]
	DOT AP/Cholesterol with protamine	pDNA	Luciferase	i.v.	–	[275]
	PEI	pDNA	TNF-alpha	i.v.	Neuroblastoma	[270]

Antibodies						
Anti-transferrin receptor single-chain antibody fragment (TfRscFv)	Liposomes (DOTAP/DOPE)	siRNA	HER-2	i.v.	Pancreatic cancer model	[196]
Antagonist G	DOT AP/HSPC/Chol/DSPE-PEG	siRNA	Bcl-2	i.v.	Small cell lung carcinoma	[216]
Single chain antibody fragment (scFv)	DOT AP/Cholesterol liposomes with protamine and hyaluronic acid	siRNA, miRNA	siRNA: c-Myc: MDM2: VEGF, miRNA-34a	i.v.	Lung metastasis	[215]
Aptamers						
PSMA-aptamer	–	siRNA	PLK1 and BCL2	i.t.	Prostate cancer xenograft	[273]
MUC-1	PEI	pDNA	Luciferase	i.t.	Lung carcinoma	[233]
Organelle-specific targeting						
MLS	DQAsomes	pDNA	–	is. mit	Not tested	[276]
MLS	PEI	pDNA	–	is. mit		[271]
Nuclear targeting ligand (PV7)	Dextran-*g*-PEI	pDNA	pEGFP	i.v.	Cervical carcinoma, liver cancer	[267]
Ku70(2)-NLS	PEI	pDNA	Luciferase	i.n.	Bioluminescence in lungs	[272]

i.v intravenous, *i.n* intranasal, *i.t* Intratumoral, *is.mit* isolated mitochondria, *DOPE* 1,2-dioleoyl-sn-glycero-3- phosphoethanolamine, *DPPC* dipalmitoylphosphatidylcholine, *DOTAP* 1,2-dioleoyl-3-trimethylammonium propane, *DSPE-PEG* 1,2-distearoryl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol) ammonium salt, *PEI* polyethyleneimine, *PLGA* poly-lactic-co-glycolide, *SNALP* stable-nucleic acid lipid nanoparticles, *HSPC* hydrogenated (Soy)-*L*-α-phosphatidylcholine, *NLS* nucleus localization sequence, *MLS* mitochondrial leader sequence

Two commercially available dendrimers are PAMAM (polyamidoamine) and PPI (polypropyleneimine) (Fig. 8.1) with ethylenediamine and butylenediamine as central core, respectively [163]. Because dendrimers are built from ABn-type monomers, each layer or "generation" of branching units (e.g., G1,G2) doubles or triples the number of peripheral functional groups. Therefore higher generations can accommodate greater payload and there are more sites available for conjugation [165]. For this reason sixth generation PAMAM is known to be an optimal gene transfection reagent (Polyfect™). These dendrimers have been employed to effectively deliver not only plasmid DNA as well as antisense oligonucleotides [166]. Additionally Polyfect™ successfully delayed tumor growth when employed for suicide cancer gene therapy [167, 168]. Other generations of PAMAM have also gained success in gene delivery. For example interleukin-10 gene complexed with G5-PAMAM was effective in improving graft survival in murine cardiac transplant model [169]. PPI dendrimers have shown high efficiency but their application is limited owing to their cytotoxicity at high charge ratios [170]. Cellular uptake of PAMAM and PPI dendrimers with net positive charges, occurs via electrostatic interactions with anionic cell surface followed by release from endosomes by the "*proton sponge*" effect [171].

Other than PPI and PAMAM, new materials have also been utilized in formulating dendrimers. For example, Ofek et al. prepared novel dendrimers using polyglycerolamine. Dendrimer of this material when complexed with luciferase-siRNA, showed significant gene silencing (85 %) within 24 h (pre-inoculated with U87-Luc human glioblastoma cells) after a single intratumoral injection in mice (dose—20 mg/kg polyglycerolamine, 5 mg/kg luciferase-siRNA). This effect lasted for 3–4 days [172].

8.4.2.4 Polymeric Nanoparticles

These nanoparticles are polymer based colloidal particles with entrapped moieties such as NAs. Nanoparticles can effectively protect NAs from enzymatic attack and more importantly, facilitate their prolong release thus making these carriers useful for the treatment of chronic ailments such as cancer and inflammatory diseases [173, 174]. Additionally nontoxic nanoparticles can be fabricated by using biocompatible and biodegradable polymers such as PLA (poly-lactic acid) and PLGA (poly-D,L-lactic-*co*-glycolic acid) (Fig. 8.1). These polymers consist of ester groups that hydrolyze and cause polymer degradation in aqueous environment, thereby releasing the entrapped material. Polymer degradation rate and hence the drug release rate can be controlled based on polymer properties such as molecular weight, co-monomer ratio, glass transition temperature, crystallinity, end groups, etc. [175, 176]. Once the nanoparticles are internalized by cells, endosomal release of entrapped NAs occurs via surface charge reversal of polymer in the nanoparticles. The ionized acidic groups of the polymer gets protonated in the low pH environment of endosomes, and interact with negatively charged endosomal lipids, thereby causing endosome destabilization [173].

PLGA is an FDA approved polymer and therefore is widely used in polymeric nanoparticles. PLGA nanoparticles can be prepared by several techniques including solvent-evaporation, nano-precipitation, solvent displacement [177], emulsion-diffusion [178]. These techniques involve use of organic solvents, vigorous stirring or homogenization. Such harsh conditions may adversely affect stability of NAs potentially lowering their activity. Additionally, limited size of nanoparticles and lack of favorable interactions between anionic polymer groups (in PLGA) and NA may result in low encapsulation efficiency. Several approaches have been adopted to overcome these challenges. As an example, high molecular weight polymers (100 kDa) have been utilized to effectively shield DNA from shear stress during the preparation process. DNA protection, in this case, was probably due to increase in solution's viscosity attributed to large polymer chains [179, 180]. Increase in nanoparticle encapsulation efficiency has been achieved by: (1) reducing sonication time during nanoparticle formulation process [181]; (2) preparing large size PLGA particles (microspheres) [182]; (3) adding NAs after nanoparticle preparation. The third approach usually results in 80–100 % encapsulation if a bridging agent is employed to associate anionic PLGA nanoparticles with anionic NAs. Several moieties such as chitosan [178], PEI [183] and cetyl-PEI [184] have been utilized as bridging agents.

8.4.2.5 Microbubbles (Ultrasound-Mediated Delivery)

Microbubbles are micron sized (2–5 μm) gas-filled bubbles formed by purging air or heavy gas (sulphur hexafluoride, perfluoropropane, perfluorobutane) into a solution of proteins, polymers or lipids [38]. Microbubbles burst upon exposure to low frequency ultrasound. Due to this feature, microbubbles can be used for site-specific gene delivery: upon in vivo delivery, microbubbles carry genes until desired site is reached. Ultrasound is then applied to this site causing release of entrapped NAs. This release usually occurs due to ultrasound-triggered cavitation at the bubble-plasma membrane interface [185]. Gene delivery efficiency is dependent upon ultrasound pressure amplitude, pulse frequency as well as duration of exposure [39].

Gene loading in microbubbles can be obtained either by entrapping NAs internally or complexing these onto the bubble surface. Entrapment would protect NAs and therefore is a good technique if naked DNA is being used [37]; however, this method offers lower loading efficiency due to the limited space. To overcome this issue, NAs can be complexed on relatively large surface area of microbubbles but NAs must be pre-complexed with cationic liposomes/polymers for enzymatic stability. For example Lentacker et al. loaded PEGylated DNA-lipoplexes onto microbubble surface via biotin-avidin-biotin linkages. These formulations showed greater transfection efficiency when compared to PEGylated lipoplexes not associated with microbubbles, in human melanoma (BLM) cells [40]. In another study, microbubbles associated lipoplexes were prepared for effective messenger RNA delivery in dendritic cells [41].

Although microbubble technology can provide efficient site-specific delivery, there are some disadvantages associated with it. For example, exposure of microbubbles to ultrasound may increase blood temperature and cause hemolysis. Additionally, ultrasound-mediated microbubble cavitation may exert mechanical pressure and cause cell damage and even cell death [186]. Despite these shortcomings, microbubbles are the only nonviral delivery carriers that can be used for targeted delivery without the use of specific targeting strategies (passive/active).

8.5 Targeted Delivery

Delivery of a therapeutic molecule directly to the desired site of action not only accelerates therapy benefits but also reduces the chances of drug side effects especially in case of toxic drugs. Additionally, there is improved patient compliance as the dose frequency is lower with targeted systems. Various nonviral delivery vectors (discussed above) have been employed to achieve targeted gene delivery using passive and/or active targeting approaches (Table 8.2).

8.5.1 Passive Targeting

Passive targeting can be achieved by regulating physicochemical parameters of the delivery vector such as size, surface charge or composition, etc. in order to increase blood circulation time usually resulting in improved accumulation in target tissues. Such an approach is specifically useful for cancer therapy since tumor cells have leaky vasculature (200–780 nm fenestrae) [187] and poor lymphatic drainage, the phenomena is known as enhanced permeability and retention (EPR) effect (Fig. 8.3). Therefore, extravasation of tumor cells is achievable with larger sized (100–200 nm) particles that are forbidden in the regions of normal tissues where there are tight junctions. However, the maximum limit for size of particles penetrating tumor tissues depends on the type of tumor. For example PEGylated liposomes of size 126 nm but not 400 nm could penetrate solid tumors, although neither of these particles was detected in healthy cells [188].

Another approach to prolong particle blood circulation time is by employing polymers such as poly-ethylene glycol (PEG) into the delivery carriers. PEGs are hydrophilic polymers that can easily “fool” macrophages, thereby preventing uptake by reticulo-endothelial system (RES). Accordingly, PEG decorated delivery vectors have delayed clearance and thus there is a greater chance of particles accumulating in the desired site [189]. Additionally, steric repulsion between the PEG chains inhibits close association of delivery vectors. Even if there is an overlap of PEG chains, a region with high osmotic pressure is created in the overlapped region that forces the solvent to enter in, and push the PEGylated nanoparticles away. In this way, PEG chains provide steric stabilization to particles, the efficiency of which

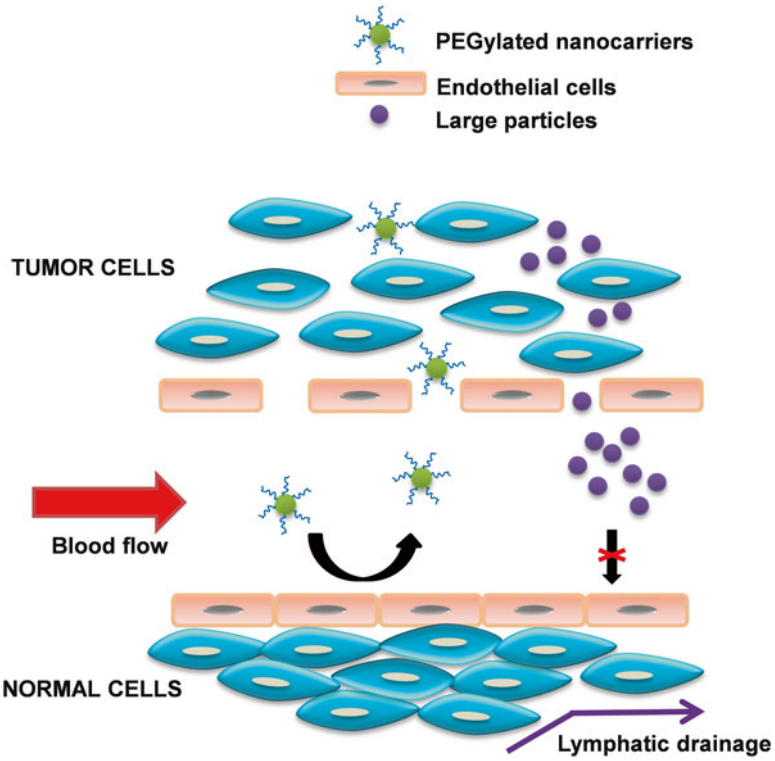

Fig. 8.3 Passive targeting approaches utilizing EPR (enhanced permeability and retention) effect in tumor cells: gene delivery systems (large sized/PEGylated) can extravasate tumor cells due to leaky vasculature and stay there for a longer time due to poor lymphatic drainage in tumor vasculature. On the other hand, large/PEGylated delivery systems are unable to penetrate normal tissues due to tight junctions in endothelial cells. However, if some particles pass through normal tissues, these get cleared off rapidly due to the lymphatic drainage

depends on the PEG chain length [190]. For these reasons, PEG is useful for long-term gene silencing. For example, mice injected with PEGylated cationic lipoplexes (SNALP) has shown efficient gene silencing of hepatitis B virus for up to 7 days with single dose [191]. Even longer silencing was achieved with single dose i.v. injection of PEGylated lipoplexes wherein tumor suppression was achieved for as long as 3 weeks [192]. Qi et al. showed efficient gene transfection with 6 % PEG-G6-PAMAM dendriplexes injected intramuscularly in neonatal mice [193]. Also William's lab prepared PEG-PEI conjugates in various molecular weight ratios (PEG:PEI), for effective delivery of antisense oligonucleotides, in vitro as well as in vivo [194]. Although PEG enhances the bio-distribution of gene delivery systems, it interferes with direct interaction of particle surface with cell membrane, thereby reducing the cellular uptake. Thus, PEG chains are usually attached to active targeting ligands that specifically bind to cell surface receptors resulting in efficient cellular internalization [195]. This approach is known as "active targeting" and has been discussed in Sect. 5.2.

8.5.2 Active Targeting

Unlike passive targeting which is a nonspecific approach, active targeting employs specific targeting moieties that bind efficiently to antigens/receptors (over-) expressed on target cell surfaces (*cell-surface targeting*). Alternately, ligands with affinity for molecules on the surface of a specific cell organelle, can be employed for *organelle-specific targeting*. Such approaches remarkably improve gene delivery efficiency and also guarantees minimal side effects. Many researchers are working on cell-surface as well as organelle specific targeting. In general, such active targeting approaches are often coupled with passive targeting approaches (PEGylation) since the combination is known to yield better results [46, 195]. Details on various active targeting approaches are as follows:

8.5.2.1 Cell-Surface Targeting

Targeting cell-surface receptors is an attractive concept to achieve specific binding and internalization using the incorporation of cell-binding ligands into delivery carriers. These ligands bind particularly to certain receptors that are selectively expressed or overexpressed on the surface of diseased cells when compared to normal cells. For example tumor cells are fast growing and therefore have greater nutritional requirements compared to normal cells. Consequently, these have overexpressed cell surface receptors required for uptake of nutritional agents such transferrin receptor for iron [196], glycosylated receptor for carbohydrates [197], and folate receptor for folic acid [198]. Accordingly, incorporation of such moieties into nonviral vectors may facilitate rapid and efficient accumulation at the target site. Uptake of cell-surface targeted delivery systems occurs via receptor-mediated endocytosis (RME) as illustrated in Fig. 8.4. Following are the examples of targeting ligands used for cell-surface targeting (see also Table 8.2):

Small Molecules

Folate (folic acid) has a high binding affinity for folate receptors whose expression is upregulated in many tumors [199, 200]. Folate is a preferable ligand due to its small size, non-immunogenicity, temperature and pH stability (over a broad range), and therefore has been widely used in targeted gene delivery systems. Kim et al. showed that folate-PEG-PEI/shRNA complexes efficiently inhibited green fluorescent protein (GFP) expression in KB cells (tumor cells). At N/P ratio of 24/1, folate polyplexes showed 90 % silencing, whereas it was only 60 % in case of non-folate polyplexes [201]. Yoshizawa et al. used PEGylated-folate lipoplexes for efficient knockdown of HER-2 in KB cells [198]. First in vivo "proof of concept" for folate targeting was demonstrated by Hofland et al. wherein folate-PEGylated lipoplexes showed efficient accumulation in tumors when compared to non-folate PEGylated lipoplexes [195].

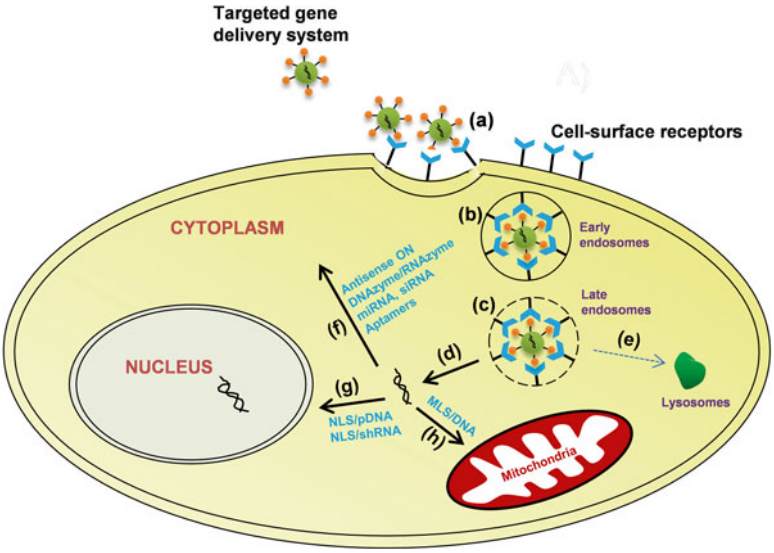

Fig. 8.4 Active targeting approaches for targeted delivery of NAs: Uptake of these particles occurs via receptor-mediated endocytosis (RME). (**a**) Targeting ligand in a gene delivery system, assist in *cell-surface targeting* upon specifically binding to cell-surface receptors (transferrin/small molecule/peptide/antibody/aptamer). (**b**) Early endosomes mature into (**c**) late endosomes. (**d**) At this point delivery carrier must assist in endosomal release of NAs which would otherwise (**e**) degrade in lysosomes. (**f**) Upon endosomal release, certain NAs (antisense, DNAzyme/RNAzyme, aptamers, miRNA and siRNA) remain in cytoplasm and function here. (**g**) Others including pDNA/shRNA with/without NLS, penetrate into the nucleus, whereas (**h**) pDNA with MLS enter mitochondria ("*g*" *and* "*h*" *represent organelle-specific targeting*). *NLS* nucleus localization sequence. *MLS* mitochondrial leader sequence

Asialoglycoprotein receptor (ASGP-R) is expressed predominantly in liver cells and has affinity for a variety of carbohydrates including lactose, galactose, and N-acetylamine. Accordingly, these moieties incorporated into nonviral vectors can be useful for liver [202]. For example DNA lipoplexes prepared with galactosylated liposomes showed significantly higher luciferase expression in HepG2 cells when compared to unmodified liposomes [203]. N-acetylgalactoseamine conjugated -poly(vinyl ether) successfully silenced expression of apolipoprotein and peroxisome proliferator-activated receptor alpha genes, in mouse liver [204]. In a recent study, systemic administration of galactosylated-liposome siRNA nanoparticles markedly helped reduce liver injury in mice infected with hepatitis [205].

Cholesterol and its derivatives have also been used for targeted gene delivery. For example, cholesterol conjugated-apolipoproteinB-siRNA has been utilized to efficiently downregulate apo-B gene expression (57 ± 6 % compared to control), that is otherwise responsible for hypercholesterolemia [206].

Anisamide containing drugs have been shown to have good binding affinity for sigma receptors that are overexpressed in many tumors. Accordingly, anisamide containing delivery systems have been utilized to achieve tumor specific targeting.

For example anisamide conjugated PEGylated liposomes have shown efficient luciferase silencing compared to non-anisamide lipoplexes, in lung cancer cells (H-460) [207]. Guo et al. formulated anisamide-PEG-cyclodextrin nanoparticles for siRNA delivery to prostate tumors in mice. After 22 days of injection tumor volume reduced significantly with anisamide-PEG-cyclodextrin siRNA complexes compared to PEG-cyclodextrin siRNA complexes [208].

Transferrin

Transferrin, an iron transporting serum glycoprotein, upon binding to the transferrin receptor can be internalized efficiently into the cells (via RME-Fig. 8.4) and through this physiological process, iron is delivered into the cells. Although the transferrin receptor is expressed on almost all cell types, it is overexpression in many rapidly dividing tumors [196]. Accordingly, transferrin bedecked nonviral carriers have been proved to be effective in gene therapy. For example, Kirchies et al. showed tumor targeting with transferrin-PEI/DNA when injected systemically in mice growing neuro2 tumors [209]. Huang et al. demonstrated successful brain targeting of luciferase DNA using transferrin-PEG-PAMAM dendrimers upon intravenous administration in mice [210]. Transferrin-conjugated PEGylated liposomes loaded with anti-BCR-ABL siRNA have been utilized for the treatment of chronic myeloid leukemia in K562 and LAMA-84 cells [211]. A transferrin-containing multicomponent siRNA formulation (CALAA-01) is in Phase I clinical trials [212] (Table 8.1).

Antibodies or Antibody Fragments

Specific antibodies for certain cell-surface markers can also be used for targeting. For example a monoclonal antibody directed to the CD3 surface marker in human T-cell leukemia enabled efficient gene delivery in vitro [213]. Antibodies have two main fragments: Fc (constant) and Fab (variable). The Fc fragment is responsible for immunogenicity of antibody since it has multiple compliment activation and macrophage binding sites, whereas Fab fragment is non-immunogenic and has antigen binding sites which are responsible of its specific interaction with the antigen. Accordingly, use of antibody fragments has become more common than the whole antibody. For examples, ErbB2, a tumor marker that is highly upregulated in many human breast and prostate cancers, was targeted with a delivery system containing a single-chain antibody [214]. Other examples include the successful use of liposome-polycation-hyaluronic acid modified with single chain antibody fragment (scFv) for dual delivery of siRNA and miRNA when administered systemically in lung-metastasis bearing mice [215]. Cell-type-specific targeting using antibodies was demonstrated by Song et al. In this work, a fusion protein based on protamine coding sequence (F105b) was conjugated at the C terminus to Fab fragment of an HIV-1 envelope antibody. When antibody-F105b siRNA complexes were injected

intravenously in mice inoculated with HIV envelope expressing B16 melanoma cells, silencing was specifically observed in HIV-1 expressing cells. When the antibody (HIV-1 envelope) in such a delivery system, was replaced with ErbB2 single-chain antibody (ErbB2-protamine-siRNA), siRNA was observed to accumulate specifically in ErbB2-expressing cancer cells [47]. Pirollo et al. used transferrin antibody targeted lipoplexes. Three intravenous doses showed knockdown of HER-2 gene in mice tumors for 30 days [196]. Santos prepared antagonist G associated targeted PEGylated liposomes for downregulation of Bcl-2 in SCLC SW2 tumor cells [216]. Recent efforts are focusing on antibody-based targeting for treatment/diagnosis of brain cancer. For example Shen et al. prepared PEG-PEI superparamagnetic iron oxide nanoparticles modified with GD2 single chain antibody which binds specifically to neuroblastoma cell-surface receptors. These nanoparticles complexed with Bcl-2 siRNA showed efficient silencing of Bcl-2 gene in human neuroblastoma cells compared to non-targeted nanoparticles (no antibody). Additionally significant apoptosis and suppression of tumor growth was observed with these targeted nanoparticles [217].

Peptides

Peptide-based targeting of nonviral delivery vectors has become possible because of our increased understanding of the discrete peptide sequences of proteins involved in cell–cell and effector–cell interactions. Various peptides have been identified as ligands that bind to receptors present specifically in diseased cells [218–222]. Accordingly, delivery systems incorporated with peptide-based ligands can facilitate efficient targeted delivery of genes to the diseased site. For example, Moreira et al. prepared tumor targeted delivery vectors using a hexapeptide analogue, antagonist G, of the neurotransmitter substance P, which binds to receptors on the surface of the small cell lung cancer (SCLC) and blocks the action of multiple neuropeptides, such as vasopressin, gastrin releasing peptide, and bradykinin [223]. Asai et al. isolated peptides specific for tumor angiogenic vasculature by in vivo bioplanning of a phage-displayed peptide library [224]. Phage fused MCF-7 cell specific peptide "DMPGTVLP" incorporated into liposomes, has been utilized for efficient siRNA delivery to breast cancer (MCF-7) cells [218]. In another study, RGD (arginine-glycine-aspartic acid tripeptide) associated PEGylated nanoparticles injected intravenously, have shown significant tumor growth inhibition in nude mice bearing human glioma U87 xenografts, when compared to non-targeted siRNA complexes [221]. Streipe et al. prepared liposomal siRNA delivery systems modified with RGD-peptide for specific recognition by integrin receptors in alveolar rhabdomyosarcoma cells. These targeted systems showed improved silencing over the unmodified ones [220]. Arginine has been used as a targeting ligand by Zhang et al. where a PEGylated octamer of arginine (R8) was decorated on siRNA-loaded cationic liposomes for efficient silencing of HDM2 gene (human double minute gene2) [222]. Kim et al. synthesized poly-L-arginine conjugated PEG (PLR-PEG) and prepared liposomes using the cationic lipid DOTAP, fusogenic lipid DOPE,

cholesterol, and PLR-PEG. Such arginine conjugated targeted liposomes were utilized to knockdown GFP in H4II-E and HepG2 cells. Accordingly, it was found that PLG-PEG liposomes were 30 % more efficient than conventional liposomes (non-PEGylated and non-targeted) at N/P ratio of 30:1 [219]. Recently, Siould et al. showed efficient silencing of survivin (an important gene for cancer cell survival) using siRNA conjugated to gastrin-releasing peptide (GRP), which binds specifically to GRP receptors on the surface of breast cancer cells [225].

Other than their application in cell-surface targeting, peptides have also been employed to improve cellular uptake, endosomal escape and/or nucleus localization gene delivery vectors. A majority of peptides show pH-dependent fusogenic and endosomolytic (endosomal escape) activity and are believed to mimic virus like journey into the cell. These peptides also called cell-penetrating peptides (CPPs) are random coil at pH 7.0 but undergo a conformational change into an amphipathic alpha-helix at endosomal pH. This conformational change induces the fusion and lysis of endosomal membrane causing the release of entrapped genes [226]. Certain examples of CPPs include TAT, penetratin, transportan, and INF [227]. Torchillin et al. have shown TAT-liposomes to be more effective and less cytotoxic than liposome control when injected subcutaneously in mice inoculated with Lewis lung carcinoma tumors [228]. Penetratin or transportan have been conjugated to siRNA for knockdown of many target proteins such as luciferase and green fluorescent protein in several cell lines [95]. Since direct conjugation of these peptides may affect NA bioactivity, recent work is focusing on non-covalent association of peptide with DNA/siRNA. For example Simeoni et al. prepared MPG peptide (sequence derived from HIV-1 and NLS) that associates with siRNA via electrostatic and hydrophobic interactions and helps with efficient siRNA delivery in vitro as well as in vivo [44, 229]. Deshayes et al. prepared another peptide (CADY) and formed stable nanostructures with siRNA for potent delivery in primary cells [230]. The nucleus localization potential of peptides has been discussed in details in Sect. 5.2.2.1.

Aptamers

Aptamers are NA (Sect. 2.3) or peptide-based molecules that bind specifically to a particular target moiety. As more and more surface molecules are being identified as biomarkers for a particular tumor, aptamer-based cell-surface targeted delivery is gaining importance. For example PSMA (prostrate-specific membrane antigen) molecule occurs predominant on the surface of human prostate cancer cells and therefore incorporation of PSMA in gene delivery systems have been successfully employed for the treatment of prostate cancer [231, 232]. Kurosaki and co-workers developed MUC-1 aptamer containing PEI/pDNA complexes that showed higher transfection efficiency in lung cancer cells (~threefold greater) and tumor-bearing mice (~4-fold greater), compared to non-targeted polyplexes [233]. AS1411 is a DNA aptamer used in the treatment of acute myeloid leukemia. AS1411 targets

cancer cell surface protein, nucleolin, gets internalized and inhibits nuclear factor-κB [234]. AS1411 also destabilizes mRNA of the anti-apoptotic protein, B-cell lymphoma protein 2 (BCL-2) [235]. Other examples of aptamer-targeting are included in Table 8.2.

8.5.2.2 Organelle-Specific Targeting

For certain therapeutic molecules such as pDNA (and shRNA), it is not sufficient to accumulate in cytoplasm. These moieties further need to enter specific cell organelles such as nucleus, mitochondria *etc*., in order to transcribe and eventually express therapeutic proteins. Penetration of NAs into an organelle is challenging due to their large size and hydrophilicity. However, various (see below) strategies have been adopted to design gene delivery systems for efficient accumulation in a particular cell organelle.

Nucleus Targeting

After endosomal release, pDNA/shRNA molecules need to find their way into the nucleus in order to employ therapeutic benefits (Fig. 8.4). Nuclear transfer of pDNA/shRNA usually occurs either via passive entry during cell division and/or by active process via nuclear pores [147, 236]. Besides, non-nuclear pore pathways for DNA nuclear import (in cells that are not undergoing active division) have been reported. For example, it has been speculated that DNA-PEI complexes may enter the nucleus via non-nuclear-pore pathways such as fusion with nuclear membrane phospholipids [237]. Although the entry of these polyplexes into the nucleus may be an effective method of delivery of DNA to the nucleus, the potential for interaction of these polycations with host genes is a significant concern. Accordingly, focus is towards improving nuclear uptake of DNA via nuclear-pore complex (NPC) especially in non-dividing or slowly dividing cells. NPC has a molecular sieve function allowing small molecules (up to 50 KDa) to diffuse through, whereas larger molecules require an active transport mechanism to pass [238]. Active transport of macromolecules (e.g., cytoplasmic proteins) is controlled by short peptides known as nuclear localization sequence (NLS) [239]. NLS has affinity for the proteins of NPC like importin-α. Activated importin-α further activates importin-ß protein which then helps in opening the nuclear-pore complex (see Fig. 8.5). During this active transport the nuclear pore complex opens from ca. 10 to ca. 40 nm and thus can accommodate plasmid DNA, which in its condensed form has a diameter of ca. 25 nm [240, 241]. NLS associated DNA can therefore be utilized to enhance DNA import to nucleus.

NLSs are usually characterized by one short peptide sequence containing many lysine and arginine residues [242]. The first NLS sequence (PKKKRKV) was derived from the tumor antigen, simian virus-40 [243]. Various NLSs have been

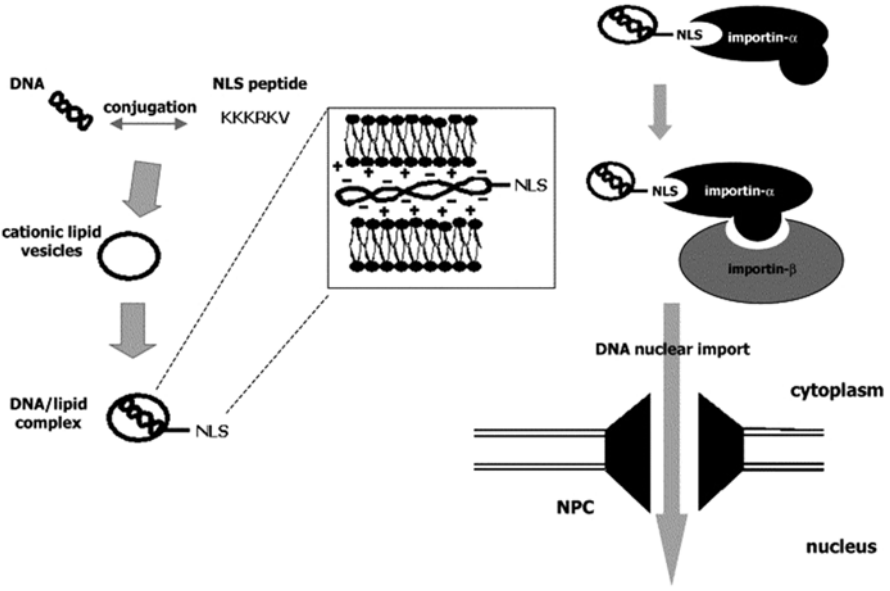

Fig. 8.5 Schematic representation of the nuclear localising peptide-conjugated DNA nuclear import mechanism. A well-defined nuclear targeting peptide signal sequence is conjugated to the DNA. Intracellular delivery of the DNA–NLS conjugate is mediated by formation of lipoplexes via the electrostatic interaction of negatively charged DNA molecules with cationic lipid molecules. In the cytoplasm, the importin-α transport receptor binds the DNA–NLS conjugate and together with importin-β mediates interaction with the nuclear pore complex to translocate the import complex into the nucleus. Reproduced from ref. [239]

attached with plasmid DNA via covalent or non-covalent association to facilitate nuclear uptake [24, 54, 244, 245]. However, both the approaches have their own challenges. For example, it has been shown that non-covalent association may result in separation of NLS from pDNA in the extracellular environment, if the binding is not strong enough [24, 246]. With respect to direct conjugation approach, NLS-pDNA conjugates have shown reduced transfection efficiency compared to pDNA alone [247]. This is apparently due to the requirement of several NLS molecules/plasmid to cause efficient nuclear uptake since some of the cationic NLSs could neutralize charge on DNA [248, 249]. However, conjugation of multiple NLSs to pDNA may alter its conformation, thereby impeding DNA activity [250]. To combat this issue, spacer moieties between NLS and DNA, such as oligonucleotides [251], psoralen [54] or PEGs [252] have been used. Alternately, NLS has been incorporated into the nonviral vectors, instead of DNA [246, 253]. For example, efficient nuclear uptake of PLGA nanoparticles [254] and HPMA copolymer [54, 255] has been reported when the NLS is attached directly to carrier (nanoparticles/polymer). NLS has also been conjugated to quantum dot (QD) nanoparticles to achieve rapid nuclear uptake [256, 257]. A recent review covers many examples for NLS associated targeted gene delivery systems [258].

Mitochondrial Targeting

Mitochondria are membrane-bound organelles whose main function is to produce adenosine triphosphate (ATP), the chemical source of energy. Mitochondria also regulate cell signaling, cell proliferation and apoptosis [259, 260]. Genetic mutation (point mutation, deletions, missense) of mitochondrial DNA (mtDNA) may cause mitochondrial dysfunction leading to metabolic disorders, neurodegenerative diseases and even tumors [48]. Various efforts are being made to cure genetic defects in mtDNA by delivering correct copy of DNA into the mitochondria [50–53].

Researchers have identified certain features which when incorporated into delivery vectors, could facilitate efficient mitochondrial delivery. For example, mitochondria have a very high inner membrane potential (180–200 mV) [49] and therefore attract molecules with high cationic charge density. These molecules need to be amphiphilic so that these can be accumulated onto the mitochondrial surface. For example, vesicles of an amphiphilic cationic surfactant, dequalinium chloride (DQA), were shown to efficiently transport DNA onto the mitochondrial surface [50, 51]. Unlike other cationic lipids (such as DOTAP, DOTMA), DQA release most of the DNA upon encountering cardiolipin-rich anionic membrane of mitochondria when compared to lesser anionic inner-cytosolic membrane. Although with DQAs, DNA could be delivered efficiently onto the surface of mitochondria, DNA internalization was minimal [51]. This issue was resolved with DNA conjugated to mitochondria targeting peptide also known as mitochondrial leader sequence (MLS) (Fig. 8.4) [50]. MLS has also been used with low molecular weight PEI (2,000 Da) to effectively deliver DNA to mitochondria in a cell-free assay [52]. A recent review has included many other examples for effective mitochondrial delivery of NAs using various delivery systems [53].

8.6 Multifunctional Nano-Carriers

After tremendous research on nonviral delivery carriers, it has been concluded that it is more beneficial to use combinational carriers (more than one type of vector, PEG, targeting ligands) compared to when a single approach is employed. This is evident from various examples in the literature some of which have been enumerated here: PLGA nanoparticles entrapping PEI-siRNA complexes showed greater efficiency in vivo compared to non-PEI containing PLGA nanoparticles [184]. In another strategy, chitosan-siRNA encapsulated in PLGA nanofibers have shown to cause 50 % gene silencing and no significant toxicity (48 h post-transfection) in non-small-cell lung carcinoma cells (H1299) [261]. Xun et al. prepared biodegradable PEI conjugated with small hydrophobic lipid molecules. These conjugates showed high transfection efficiency and low cytotoxicity in human non-small-cell lung carcinoma when compared to PEI (25 kDa) alone [262]. Rui et al, prepared PEI condensed siRNA nanocomplexes and coated these with lipid (egg phosphatidylcholine) and human apolipoprotein A-I, to achieve effective and safe delivery of

siRNA in liver cancer (hepatoma) cells [263]. Dextran grafted PEI–DNA complexes incorporated with nuclear localization sequence (Sv7) showed efficient GFP expression in vitro (HepG2, HeLa, 293 T cells) as well as in vivo (mice xenograft model) [264]. A novel triblock copolymer composed of PEG, PLGA, and PLL (mPEG-PLGA-PLL) has shown gene silencing greater than Lipofectamine™ with no cytotoxicity in human lung cancer cells (SPC-A1) [265]. Oishi and coworkers prepared a triblock polymer conjugated to a targeting ligand (lactose), PEG, and siRNA on three different sites for efficient cellular uptake as well as endosomolytic activity [266]. Owing to their great potential and proven success, such multifunctional carriers have become a favorite of researchers working on the development of efficient gene delivery systems.

8.7 Summary and Future Perspectives

Targeted NA delivery is a challenge that requires the development of delivery vectors that specifically direct NAs to the desired site without adversely affecting healthy cells. As evident from various examples covered in this chapter, several labs have developed safe and efficient targeted gene delivery systems by employing active and/or passive approaches. Despite a remarkable success of these products in preclinical studies, there are only two targeted gene delivery products currently in clinical trials (Table 8.1 and [57]). This could apparently be due to product failure either in the late-stage preclinical (primates) or early-stage clinical trials. Such failures could be the result of wrong selection on animal models (preclinical stage), scale-up issues at industrial scale, or inter-patient variability (pharmacogenomics). Therefore, much work needs to be done to fill this gap between preclinical and clinical stages in order to have numerous targeted nucleic acid products in the clinics and eventually in the market.

References

1. Yei S et al (1994) Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo vector administration to the lung. Gene Ther 1(3):192–200
2. Aiuti A et al (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 296(5577):2410–2413
3. Cavazzana-Calvo M et al (2004) Gene therapy for severe combined immunodeficiency. Annu Rev Med 56(1):585–602
4. Ganly I, Soutar DS, Kaye SB (2000) Current role of gene therapy in head and neck cancer. Eur J Surg Oncol 26(4):338–343
5. Wahlfors T et al (2006) In vivo enhancement of herpes simplex virus thymidine kinase/ganciclovir cancer gene therapy with polyamine biosynthesis inhibition. Int J Cancer 118(11):2907–2910
6. Baker AH (2002) Development and use of gene transfer for treatment of cardiovascular disease. J Card Surg 17(6):543–548

7. Dishart KL et al (2003) Gene therapy for cardiovascular disease. J Biomed Biotechnol 2003(2):138–148
8. Barkats M et al (1998) Adenovirus in the brain: recent advances of gene therapy for neurodegenerative diseases. Prog Neurobiol 55(4):333–341
9. Mark HT et al (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 11(5):551–555
10. National Institute of Health (1990) The first gene therapy trial. http://history.nih.gov/exhibits/genetics/sect4.htm#2. Accessed 5 Jul 2013
11. The Human Genome Management Information System (HGMIS) (2011) About the human genome project: what is the human genome project. http://www.ornl.gov/sci/techresources/Human_Genome/project/about.shtml. Accessed 5 Jul 2013
12. Gene Therapy Clinical Trials Worldwide 2013. http://www.wiley.com/legacy/wileychi/genmed/clinical/. Accessed 4 Mar 2013
13. Raty JK et al (2010) Gene therapy: the first approved gene-based medicines, molecular mechanisms and clinical indications. Curr Mol Pharmacol 1(1):13–23
14. Richards S (2012) Gene therapy arrives in Europe. The Scientist 1(1):2
15. Masuda T, Akita H, Harashima H (2005) Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: the use of a nuclear transfer score. FEBS Lett 579(10):2143–2148
16. McAllister DV, Allen MG, Prausnitz MR (2000) Microfabricated microneedles for gene and drug delivery. Ann Rev Biomed Eng 2(1):289–313
17. Yang N-S et al (1990) In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci 87(24):9568–9572
18. Molnar MJ et al (2004) Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles. Mol Ther 10(3):447–455
19. Choung S et al (2006) Chemical modification of siRNAs to improve serum stability without loss of efficacy. Biochem Biophys Res Commun 342(3):919–927
20. Hall AH et al (2004) RNA interference using boranophosphate siRNAs: structure-activity relationships. Nucleic Acids Res 32(20):5991–6000
21. Layzer JM et al (2004) In vivo activity of nuclease-resistant siRNAs. RNA 10(5):766–771
22. Parrish S et al (2000) Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol Cell 6(5):1077–1087
23. Amarzguioui M et al (2003) Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res 31(2):589–595
24. Escriou V et al (2003) NLS bioconjugates for targeting therapeutic genes to the nucleus. Adv Drug Deliv Rev 55(2):295–306
25. Walther W, Stein U (2000) Viral vectors for gene transfer. Drugs 60(2):249–271
26. Yang Y, Ertl HC, Wilson JM (1994) MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1(5):433–442
27. Yang Y et al (1995) Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J Virol 69(4):2004–2015
28. Ikebe M et al (2009) Lipopolysaccharide (LPS) increases the invasive ability of pancreatic cancer cells through the TLR4/MyD88 signaling pathway. J Surg Oncol 100(8):725–731
29. Kedmi R, Ben-Arie N, Peer D (2010) The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. Biomaterials 31(26):6867–6875
30. Lv H et al (2006) Toxicity of cationic lipids and cationic polymers in gene delivery. J Control Release 114(1):100–109
31. Srinivasan C, Burgess DJ (2009) Optimization and characterization of anionic lipoplexes for gene delivery. J Control Release 136(1):62–70
32. Kapoor M, Burgess DJ (2012) Physicochemical characterization of anionic lipid-based ternary siRNA complexes. Biochim Biophys Acta 1818(7):1603–1612

33. Kapoor M, Burgess DJ (2012) Efficient and safe delivery of siRNA using anionic lipids: formulation optimization studies. Int J Pharm 432(1–2):80–90
34. Patil SD, Rhodes DG, Burgess DJ (2004) Anionic liposomal delivery system for DNA transfection. AAPS J 6(4):e29
35. Lee RJ, Huang L (1996) Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J Biol Chem 271(14):8481–8487
36. Kapoor M, Burgess D (2013) Cellular uptake mechanisms of novel anionic siRNA lipoplexes. Pharm Res 30(4):1161–1175
37. Frenkel PA et al (2002) DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection in vitro. Ultrasound Med Biol 28(6):817–822
38. Cool SK et al. (2013) Enhancing nucleic acid delivery with ultrasound and microbubbles. In: Nanotechnology for nucleic acid delivery. Springer, New York, p 195–204
39. Rahim A et al (2006) Physical parameters affecting ultrasound/microbubble-mediated gene delivery efficiency in vitro. Ultrasound Med Biol 32(8):1269–1279
40. Lentacker I et al (2009) Ultrasound exposure of lipoplex loaded microbubbles facilitates direct cytoplasmic entry of the lipoplexes. Mol Pharm 6(2):457–467
41. De Temmerman M-L et al (2011) mRNA-Lipoplex loaded microbubble contrast agents for ultrasound-assisted transfection of dendritic cells. Biomaterials 32(34):9128–9135
42. Ogris M et al (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther 6(4):595–605
43. Harris TJ et al (2010) Tissue-specific gene delivery via nanoparticle coating. Biomaterials 31(5):998–1006
44. Simeoni F et al (2003) Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. Nucleic Acids Res 31(11): 2717–2724
45. Pulford B et al (2010) Liposome-siRNA-peptide complexes cross the blood-brain barrier and significantly decrease PrP on neuronal cells and PrP in infected cell cultures. PLoS ONE 5(6):e11085
46. Arima H et al (2012) Potential use of folate-polyethylene glycol (PEG)-appended dendrimer (G3) conjugate with beta-cyclodextrin as DNA carriers to tumor cells. Cancer Gene Ther 19(5):358–366
47. Song E et al (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol 23(6):709–717
48. Heller A, Brockhoff G, Goepferich A (2012) Targeting drugs to mitochondria. Eur J Pharm Biopharm 82(1):1–18
49. Vaidya B et al (2009) Targeted nucleic acid delivery to mitochondria. Curr Gene Ther 9(6):475–486
50. D'Souza GGM, Boddapati SV, Weissig V (2005) Mitochondrial leader sequence-plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria. Mitochondrion 5(5):352–358
51. D'Souza GGM et al (2003) DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells. J Controlled Release 92(1–2):189–197
52. Choi JS et al (2006) Low molecular weight polyethylenimine-mitochondrial leader peptide conjugate for DNA delivery to mitochondria. Bull Kor Chem Soc 27(9):1335–1340
53. Edeas M, Weissig V (2013) Targeting mitochondria: strategies, innovations and challenges: the future of medicine will come through mitochondria. Mitochondrion, 2013. (In Press) Corrected proof: p. http://dx.doi.org/10.1016/j.mito.2013.03.009
54. Yoo HS, Jeong SY (2007) Nuclear targeting of non-viral gene carriers using psoralen-nuclear localization signal (NLS) conjugates. Eur J Pharm Biopharm 66(1):28–33
55. Yu H et al (2011) Effects of MDM2 promoter polymorphisms and p53 codon 72 polymorphism on risk and age at onset of squamous cell carcinoma of the head and neck. Mol Carcinog 50(9):697–706

56. Fioretti D et al (2010) DNA vaccines: developing new strategies against cancer. J Biomed Biotechnol 2010:1–16
57. Clinical Trials. www.clinicaltrial.gov. Accessed 5 Jul 2013
58. Malecki M (2012) Frontiers in suicide gene therapy of cancer. J Genet Syndr Gene Ther 2012(3):e114
59. Kim JH et al (2012) Therapeutic effect of genetically modified human neural stem cells encoding cytosine deaminase on experimental glioma. Biochem Biophys Res Commun 417(1):534–540
60. Shen L-Z et al (2002) Specific CEA-producing colorectal carcinoma cell killing with recombinant adenoviral vector containing cytosine deaminase gene. World J Gastroenterol 8(2):270–275
61. Ueda K et al (2001) Carcinoembryonic antigen-specific suicide gene therapy of cytosine deaminase/5-fluorocytosine enhanced by the cre/loxP system in the orthotopic gastric carcinoma model. Cancer Res 61(16):6158–6162
62. Dass CR (2004) Deoxyribozymes: cleaving a path to clinical trials. Trends Pharmacol Sci 25(8):395–397
63. Ke X, Yang Y-c, Hong S -l (2011) EBV-LMP1-targeted DNAzyme restrains nasopharyngeal carcinoma growth in a mouse C666-1 xenograft model. Med Oncol 28(1):326–332
64. Lipkin D, Talbert PT, Cohn M (1954) The mechanism of the alkaline hydrolysis of ribonucleic acids. J Am Chem Soc 76(11):2871–2872
65. Diener JL et al (2009) Inhibition of von Willebrand factore-mediated platelet activation and thrombosis by the anti-von Willebrand factor A1-domain aptamer ARC1779. J Thromb Haemost 7(7):1155–1162
66. Ni X et al (2011) Nucleic acid aptamers: clinical applications and promising new horizons. Curr Med Chem 18(27):4206
67. Zhou J, Rossi JJ (2012) Therapeutic potential of aptamer-siRNA conjugates for treatment of HIV-1. BioDrugs 26(6):393–400
68. Dias N, Stein CA (2002) Antisense oligonucleotides: basic concepts and mechanisms. Mol Cancer Ther 1(5):347–355
69. Aartsma-Rus A, van Ommen G-JB (2009) Progress in therapeutic antisense applications for neuromuscular disorders. Eur J Hum Genet 18(2):146–153
70. Atlantic Healthcare (2010): Announces it is commencing manufacture and international supply of Alicaforsen for inflammatory bowel disease under named patient supply regulations http://www.atlantichc.com/newsdocs/20100519.pdf.
71. Wahid F et al (2012) MicroRNAs: synthesis, mechanism, function, and recent clinical trials. Biochim Biophys Acta 1803(11):1231–1243
72. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2):281–297
73. Bader AG (2012) miR-34a microRNA replacement therapy is headed to the clinic. Front Genet 3(120):1–9
74. Pillai RS, Bhattacharyya SN, Filipowicz W (2007) Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 17(3):118–126
75. Alvarez JP et al (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. Plant Cell 18(5):1134–1151
76. Ma JB et al (2005) Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature 434(7033):666–670
77. Elbashir SM et al (2001) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J 20(23):6877–6888
78. Haley B, Zamore PD (2004) Kinetic analysis of the RNAi enzyme complex. Nat Struct Mol Biol 11(7):599–606
79. Kapoor M, Burgess DJ, Patil SD (2012) Physicochemical characterization techniques for lipid based delivery systems for siRNA. Int J Pharm 427(1):35–57

80. Behlke MA (2008) Chemical modification of siRNAs for in vivo use. Oligonucleotides 18(4):305–319
81. Grillone LR, Lanz R (2001) Fomivirsen. Drugs Today 37(4):245
82. Jabs DA, Griffiths PD (2002) Fomivirsen for the treatment of cytomegalovirus retinitis. Am J Ophthalmol 133(4):552–556
83. Manoharan M (2004) RNA interference and chemically modified small interfering RNAs. Curr Opin Chem Biol 8(6):570–579
84. Hornung V et al (2005) Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med 11(3):263–270
85. Gorden KB et al (2005) Synthetic TLR agonists reveal functional differences between Human TLR7 and TLR8. J Immunol 174(3):1259–1268
86. Diebold SS et al (2006) Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. Eur J Immunol 36(12):3256–3267
87. Karikó K et al (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23(2):165–175
88. Kim JY et al (2007) Immune activation by siRNA/liposome complexes in mice is sequence-independent: lack of a role for Toll-like receptor 3 signaling. Mol Cells 24(2):247–254
89. Robbins M et al (2007) 2′-O-methyl-modified RNAs act as TLR7 antagonists. Mol Ther 15(9):1663–1669
90. Cekaite L et al (2007) Gene expression analysis in blood cells in response to unmodified and 2′-modified siRNAs reveals TLR-dependent and independent effects. J Mol Biol 365(1):90–108
91. Saunders LR, Barber GN (2003) The dsRNA binding protein family: critical roles, diverse cellular functions. FASEB J 17(9):961–983
92. Kato H et al (2005) Cell type-specific involvement of RIG-I in antiviral response. Immunity 23(1):19–28
93. Oivanen M, Kuusela S, Lennberg H (1998) Kinetics and mechanisms for the cleavage and isomerization of the phosphodiester bonds of RNA by bronsted acids and bases. Chem Rev 98(3):961–990
94. Huber R, Fiebig T, Wagenknecht H-A (2003) Pyrene as a fluorescent probe for DNA base radicals. Chem Commun 15:1878–1879
95. Muratovska A, Eccles MR (2004) Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett 558(1–3):63–68
96. Oishi M et al (2007) Enhanced growth inhibition of hepatic multicellular tumor spheroids by lactosylated poly(ethylene glycol)-siRNA conjugate formulated in PEGylated polyplexes. ChemMedChem 2(9):1290–1297
97. Moschos SA et al (2007) Lung delivery studies using siRNA conjugated to TAT(48-60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. Bioconjug Chem 18(5):1450–1459
98. Chiu Y-L et al (2004) Visualizing a correlation between siRNA localization, cellular uptake, and RNAi in living cells. Chem Biol 11(8):1165–1175
99. Meade BR, Dowdy SF (2007) Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. Adv Drug Deliv Rev 59(2–3):134–140
100. Thrasher AJ et al (2006) Gene therapy: X-SCID transgene leukaemogenicity. Nature 443(7109):E5–E6, discussion E6–7
101. Thomas CE, Ehrhardt A, Kay MA (2003) Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 4(5):346–358
102. Felgner PL et al (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci 84(21):7413–7417
103. Kim TW et al (2000) In vivo gene transfer to the mouse nasal cavity mucosa using a stable cationic lipid emulsion. Mol Cells 10(2):142–147
104. Crook K et al (1998) Inclusion of cholesterol in DOTAP transfection complexes increases the delivery of DNA to cells in vitro in the presence of serum. Gene Ther 5(1):137–143

105. Felgner PL (1993) LipofectAMINE™ reagent: a new, high efficiency polycationic liposome transfection reagent. Focus 15:73–79
106. Kott M et al (1998) A new efficient method for transfection of neonatal cardiomyocytes using histone H1 in combination with DOSPER liposomal transfection reagent. Somat Cell Mol Genet 24(4):257–261
107. Cullis PR, De Kruijff B (1979) Lipid polymorphism and the functional role of lipids in biological membranes. Biochim Biophys Acta 559(4):399–420
108. Farhood H, Serbina N, Huang L (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim Biophys Acta 1235(2):289–295
109. Felgner JH et al (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 269(4):2550–2561
110. Hafez IM, Cullis PR (2001) Roles of lipid polymorphism in intracellular delivery. Adv Drug Deliv Rev 47(2–3):139–148
111. Hafez IM, Maurer N, Cullis PR (2001) On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther 8(15):1188–1196
112. Koltover I et al (1998) An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 281(5373):78–81
113. Burger HJ et al (1992) Paradoxical transcriptional activation of rat liver cytochrome P-450 3A1 by dexamethasone and the antiglucocorticoid pregnenolone 16 alpha-carbonitrile: analysis by transient transfection into primary monolayer cultures of adult rat hepatocytes. Proc Natl Acad Sci 89(6):2145–2149
114. Litzinger DC, Huang L (1992) Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. Biochim Biophys Acta 1113(2):201–227
115. Park HS, et al. (2004) Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kB. 3589–3593
116. Soenen SJH et al (2009) The role of nanoparticle concentration-dependent induction of cellular stress in the internalization of non-toxic cationic magnetoliposomes. Biomaterials 30(36):6803–6813
117. Zelphati O et al (1998) Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. Biochim Biophys Acta 1390(2):119–133
118. Yang JP, Huang L (1997) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther 4(9):950–960
119. Liu F et al (1997) Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. Gene Ther 4(6):517–523
120. Delepine P et al (2000) Cationic phosphonolipids as nonviral vectors: in vitro and in vivo applications. J Pharm Sci 89(5):629–638
121. Heyes J et al (2005) Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. J Control Release 107(2):276–287
122. Loisel S et al (2001) Factors influencing the efficiency of lipoplexes mediated gene transfer in lung after intravenous administration. J Liposome Res 11(2–3):127–138
123. Kapoor M et al (2011) Novel cationic lipids for safe and efficient siRNA delivery. Control Rel Soc Newslett 28(4):6–9
124. Balasubramaniam RP et al (1996) Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. Gene Ther 3(2):163–172
125. Felgner PL et al (1981) Asymmetric incorporation of trisialoganglioside into dipalmitoylphosphatidylcholine vesicles. Biochemistry 20(8):2168–2172
126. Felgner PL et al (1983) Kinetics of transfer of gangliosides from their micelles to dipalmitoylphosphatidylcholine vesicles. Biochemistry 22(7):1670–1674
127. Cameron FH et al (1999) A transfection compound series based on a versatile Tris linkage. Biochim Biophys Acta 1417(1):37–50

128. Pinnaduwage P, Schmitt L, Huang L (1989) Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells. Biochim Biophys Acta 985(1):33–37
129. Wang J et al (1998) Synthesis and characterization of long chain alkyl acyl carnitine esters. Potentially biodegradable cationic lipids for use in gene delivery. J Med Chem 41(13):2207–2215
130. Behr JP (1994) Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. Bioconjug Chem 5(5):382–389
131. Leventis R, Silvius JR (1990) Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. Biochim Biophys Acta 1023(1):124–132
132. Obika S et al (1997) A symmetrical and biodegradable cationic lipid. Synthesis and application for efficient gene transfection. Bioorg Med Chem Lett 7(14):1817–1820
133. Lee ER et al (1996) Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. Hum Gene Ther 7(14):1701–1717
134. Majeti BK et al (2004) Enhanced intravenous transgene expression in mouse lung using cyclic-head cationic lipids. Chem Biol 11(4):427–437
135. Dalby B et al (2004) Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. Methods 33(2):95–103
136. Zhao M et al (2008) Lipofectamine RNAiMAX: an efficient siRNA transfection reagent in human embryonic stem cells. Mol Biotechnol 40(1):19–26
137. Foged C, Nielsen HM, Frokjaer S (2007) Liposomes for phospholipase A2 triggered siRNA release: preparation and in vitro test. Int J Pharm 331(2):160–166
138. Halder J et al (2006) Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. Clin Cancer Res 12(16): 4916–4924
139. Lakkaraju A et al (2001) Neurons are protected from excitotoxic death by p53 antisense oligonucleotides delivered in anionic liposomes. J Biol Chem 276(34):32000–32007
140. Fillion P et al (2001) Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. Biochim Biophys Acta 1515(1):44–54
141. Patil SD, Rhodes DG, Burgess DJ (2005) Biophysical characterization of anionic lipoplexes. Biochim Biophys Acta 1711(1):1–11
142. Pack DW et al (2005) Design and development of polymers for gene delivery. Nat Rev Drug Discov 4(7):581–593
143. Eliyahu H, Barenholz Y, Domb AJ (2005) Polymers for DNA delivery. Molecules 10(1): 34–64
144. Gao X, Kim K-S, Liu D (2007) Nonviral gene delivery: what we know and what is next. AAPS J 9(1):E92–E104
145. Zauner W, Ogris M, Wagner E (1998) Polylysine-based transfection systems utilizing receptor-mediated delivery. Adv Drug Deliv Rev 30(1):97–113
146. Erbacher P et al (1996) Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. Exp Cell Res 225(1):186–194
147. Wagner E et al (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci 89(17):7934–7938
148. Midoux P, Monsigny M (1999) Efficient gene transfer by histidylated polylysine/pDNA complexes. Bioconjug Chem 10(3):406–411
149. Pichon C et al (2000) Histidylated oligolysines increase the transmembrane passage and the biological activity of antisense oligonucleotides. Nucleic Acids Res 28(2):504–512
150. Boussif O et al (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci 92(16):7297–7301
151. Liao H-W, Yau K-W (2007) In vivo gene delivery in the retina using polyethylenimine. Biotechniques 42(3):285–288

152. Fischer D et al (1999) A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 16(8):1273–1279
153. Godbey WT, Wu KK, Mikos AG (1999) Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 45(3):268–275
154. Zhao N, Roesler S, Kissel T (2011) Synthesis of a new potential biodegradable disulfide containing poly (ethylene imine)-poly (ethylene glycol) copolymer cross-linked with click cluster for gene delivery. Int J Pharm 411(1):197–205
155. Anderson DG et al (2005) Structure/property studies of polymeric gene delivery using a library of poly (I^2-amino esters). Mol Ther 11(3):426–434
156. Y-b L et al (2002) Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. Bioconjug Chem 13(5):952–957
157. Kleemann E et al (2009) Enhanced gene expression and reduced toxicity in mice using polyplexes of low-molecular-weight poly (ethylene imine) for pulmonary gene delivery. J Drug Target 17(8):638–651
158. Koping-Hoggard M et al (2001) Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. Gene Ther 8(14):1108–1121
159. Lavertu M et al (2006) High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. Biomaterials 27(27):4815–4824
160. Howard KA et al (2008) Chitosan/siRNA nanoparticle-mediated TNF-alpha knockdown in peritoneal macrophages for anti-inflammatory treatment in a murine arthritis model. Mol Ther 17(1):162–168
161. Wong K et al (2006) PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjug Chem 17(1):152–158
162. Klajnert B, Bryszewska M (2001) Dendrimers: properties and applications. Acta Biochim Pol 48(1):199–208
163. Dufes C, Uchegbu IF, Schatzlein AG (2005) Dendrimers in gene delivery. Adv Drug Deliv Rev 57(15):2177–2202
164. Aulenta F, Hayes W, Rannard S (2003) Dendrimers: a new class of nanoscopic containers and delivery devices. Eur Polym J 39(9):1741–1771
165. Liang P (2002) SAGE Genie: a suite with panoramic view of gene expression. Proc Natl Acad Sci 99(18):11547–11548
166. Kukowska-Latallo JF et al (1996) Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. Proc Natl Acad Sci 93(10):4897–4902
167. Maksimenko AV et al (2003) Optimisation of dendrimers-mediated gene transfer by anionic oligomers. J Gene Med 5(1):61–71
168. Vincent L et al (2003) Efficacy of dendrimer-mediated angiostatin and TIMP-2 gene delivery on inhibition of tumor growth and angiogenesis: In vitro and in vivo studies. Int J Cancer 105(3):419–429
169. Wang Y et al (2001) Combination of electroporation and DNA/dendrimer complexes enhances gene transfer into murine cardiac transplants. Am J Transplant 1(4):334–338
170. Malik N et al (2000) Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. J Control Release 65(1):133–148
171. Sonawane ND, Szoka FC, Verkman AS (2003) Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 278(45): 44826–44831
172. Ofek P et al (2010) In vivo delivery of small interfering RNA to tumors and their vasculature by novel dendritic nanocarriers. FASEB J 24(9):3122–3134

173. Panyam J, Labhasetwar V (2003) Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev 55(3):329–347
174. Prabha S, Labhasetwar V (2004) Nanoparticle-mediated wild-type p53 gene delivery results in sustained antiproliferative activity in breast cancer cells. Mol Pharm 1(3):211–219
175. Park TG (1994) Degradation of poly(d, l-lactic acid) microspheres: effect of molecular weight. J Control Release 30(2):161–173
176. Park TG, Lu W, Crotts G (1995) Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly (d, l-lactic acid-co-glycolic acid) microspheres. J Control Release 33(2):211–222
177. Jain RA (2000) The manufacturing techniques of various drug loaded biodegradable poly (lactide-co-glycolide) (PLGA) devices. Biomaterials 21(23):2475–2490
178. Ravi Kumar MNV, Bakowsky U, Lehr CM (2004) Preparation and characterization of cationic PLGA nanospheres as DNA carriers. Biomaterials 25(10):1771–1777
179. Wang D et al (1999) Encapsulation of plasmid DNA in biodegradable poly (D, L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. J Control Release 57(1):9–18
180. Ando S et al (1999) PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. J Pharm Sci 88(1):126–130
181. Koby G et al (2007) Poly (D, L-lactide-co-glycolide acid) nanoparticles for DNA delivery: waiving preparation complexity and increasing efficiency. Biopolymers 85(5–6):379–391
182. Meuller M et al (2012) Coencapsulation of tumor lysate and CpG-ODN in PLGA-microspheres enables successful immunotherapy of prostate carcinoma in TRAMP mice. J Control Release 162(1):159–166
183. Patil Y, Panyam J (2009) Polymeric nanoparticles for siRNA delivery and gene silencing. Int J Pharm 367(1):195–203
184. Andersen MA et al (2010) Surface functionalisation of PLGA nanoparticles for gene silencing. Biomaterials 31(21):5671–5677
185. Koch S et al (2000) Ultrasound enhancement of liposome-mediated cell transfection is caused by cavitation effects. Ultrasound Med Biol 26(5):897–903
186. Van Wamel A, Bouakaz A, de Jong N (2003) Duration of ultrasound bubbles enhanced cell membrane permeability. In: Ultrasonics. IEEE Symposium 1:917–920
187. Gaumet M et al (2008) Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. Eur J Pharm Biopharm 69(1):1–9
188. Ishida O et al (1999) Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. Int J Pharm 190(1):49–56
189. Woodle MC (1993) Surface-modified liposomes: assessment and characterization for increased stability and prolonged blood circulation. Chem Phys Lipids 64(1–3):249–262
190. Mosqueira VC et al (2001) Biodistribution of long-circulating PEG-grafted nanocapsules in mice: effects of PEG chain length and density. Pharm Res 18(10):1411–1419
191. Morrissey DV et al (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol 23(8):1002–1007
192. Akinc A et al (2009) Development of lipidoid-siRNA formulations for systemic delivery to the liver. Mol Ther 17(5):872–879
193. Qi R et al (2009) PEG-conjugated PAMAM dendrimers mediate efficient intramuscular gene expression. AAPS J 11(3):395–405
194. Lutz GJ, Sirsi SR, Williams JH (2008) PEG-PEI copolymers for oligonucleotide delivery to cells and tissues. In: Gene Ther Protocols. Springer. 433:141–150.
195. Hofland HEJ et al (2002) Folate-targeted gene transfer in vivo. Mol Ther 5(6):739–744
196. Pirollo KF et al (2007) Materializing the potential of small interfering RNA via a tumor-targeting nanodelivery system. Cancer Res 67(7):2938–2943
197. Wang Q, Dordick JS, Linhardt RJ (2002) Synthesis and application of carbohydrate-containing polymers. Chem Mater 14(8):3232–3244

198. Yoshizawa T et al (2008) Folate-linked lipid-based nanoparticles for synthetic siRNA delivery in KB tumor xenografts. Eur J Pharm Biopharm 70(3):718–725
199. Ross JF, Chaudhuri PK, Ratnam M (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. Cancer 73(9):2432–2443
200. Weitman SD et al (1992) Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. Cancer Res 52(23):6708–6711
201. Hwa Kim S et al (2005) Target-specific gene silencing by siRNA plasmid DNA complexed with folate-modified poly (ethylenimine). J Control Release 104(1):223–232
202. Pathak A, Vyas SP, Gupta KC (2008) Nano-vectors for efficient liver specific gene transfer. Int J Nanomedicine 3(1):31–49
203. Kawakami S et al (1998) Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. Biochem Biophys Res Commun 252(1):78–83
204. Rozema DB et al (2007) Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. Proc Natl Acad Sci 104(32):12982–12987
205. Jiang N et al (2012) A novel in vivo sirna delivery system specifically targeting liver cells for protection of ConA-induced fulminant hepatitis. PLoS One 7(9):e44138
206. Soutschek J et al (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432(7014):173–178
207. Li J et al (2010) Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery. J Control Release 142(3):416–421
208. Guo J et al (2012) Anisamide-targeted cyclodextrin nanoparticles for siRNA delivery to prostate tumours in mice. Biomaterials 33(31):7775–7784
209. Kircheis R et al (2001) Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther 8(1):28–40
210. Huang R-Q et al (2007) Efficient gene delivery targeted to the brain using a transferrin-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. FASEB J 21(4): 1117–1125
211. Mendonca LS et al (2010) Transferrin receptor-targeted liposomes encapsulating anti-BCR-ABL siRNA or asODN for chronic myeloid leukemia treatment. Bioconjug Chem 21(1): 157–168
212. Davis ME (2009) The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. Mol Pharm 6(3):659–668
213. Kircheis R et al (1997) Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. Gene Ther 4(5):409–418
214. Li X et al (2001) Single-chain antibody-mediated gene delivery into ErbB2-positive human breast cancer cells. Cancer Gene Ther 8(8):555–565
215. Chen Y et al (2010) Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. Mol Ther 18(9):1650–1656
216. Santos AO et al (2010) Design of peptide-targeted liposomes containing nucleic acids. Biochim Biophys Acta 1798(3):433–441
217. Shen M et al (2012) An MRI-visible non-viral vector for targeted Bcl-2 siRNA delivery to neuroblastoma. Int J Nanomedicine 7:3319–3332
218. Bedi D et al (2011) Delivery of siRNA into breast cancer cells via phage fusion protein-targeted liposomes. Nanomedicine 7(3):315–323
219. Kim HK et al (2010) Enhanced siRNA delivery using cationic liposomes with new polyarginine-conjugated PEG-lipid. Int J Pharm 392(1–2):141–147
220. Streipe S, Rossler J, Suss R (2010) Integrin- and IGF1-receptor-mediated liposomal siRNA delivery to alveolar rhabdomyosarcoma cells. Sci Pharm 78:674
221. Wang X-L et al (2009) Targeted systemic delivery of a therapeutic siRNA with a multifunctional carrier controls tumor proliferation in mice. Mol Pharm 6(3):738–746
222. Zhang C et al. (2006) siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J Control Release 112(2): 229–239

223. Moreira JN et al (2002) Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. Pharm Res 19(3):265–269
224. Asai T et al (2002) Anti-neovascular therapy by liposomal DPP-CNDAC targeted to angiogenic vessels. FEBS Lett 520(1–3):167–170
225. Sioud M, Mobergslien A (2012) Efficient siRNA targeted delivery into cancer cells by gastrin-releasing peptides. Bioconjug Chem 23(5):1040–1049
226. Plank C et al (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J Biol Chem 269(17):12918–12924
227. Martin ME, Rice KG (2007) Peptide-guided gene delivery. AAPS J 9(1):E18–E29
228. Torchilin VP et al (2003) Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposomes DNA complexes. Proc Natl Acad Sci 100(4):1972–1977
229. Simeoni F et al. (2005) Peptide-based strategy for siRNA delivery into mammalian cells. In: RNA silencing. Springer. p 251–260
230. Deshayes S et al (2012) Self-assembling peptide-based nanoparticles for siRNA delivery in primary cell lines. Small 8(14):2184–2188
231. Dassie JP et al (2009) Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. Nat Biotechnol 27(9):839–846
232. Liu H et al (1998) Constitutive and antibody-induced internalization of prostate-specific membrane antigen. Cancer Res 58(18):4055–4060
233. Kurosaki T et al (2012) Self-assemble gene delivery system for molecular targeting using nucleic acid aptamer. Gene 491(2):205–209
234. Girvan AC et al (2006) AGRO100 inhibits activation of nuclear factor-kB (NF-kB) by forming a complex with NF-kB essential modulator (NEMO) and nucleolin. Mol Cancer Ther 5(7):1790–1799
235. Soundararajan S et al (2008) The nucleolin targeting aptamer AS1411 destabilizes Bcl-2 messenger RNA in human breast cancer cells. Cancer Res 68(7):2358–2365
236. Simoes S et al (1999) Successful transfection of lymphocytes by ternary lipoplexes. Biosci Rep 19(6):601–609
237. Godbey WT, Wu KK, Mikos AG (1999) Tracking the intracellular path of poly (ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci 96(9):5177–5181
238. Suikkanen S et al (2003) Exploitation of microtubule cytoskeleton and dynein during parvoviral traffic toward the nucleus. J Virol 77(19):10270–10279
239. Munkonge FM et al (2003) Emerging significance of plasmid DNA nuclear import in gene therapy. Adv Drug Deliv Rev 55(6):749–760
240. Pante N, Fahrenkrog B, Aebi U (1998) Molecular dissection of nuclear pore complex structure and nucleocytoplasmic transport. Biol Cell 90:275–276
241. Pante N, Kann M (2002) Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. Mol Biol Cell 13(2):425–434
242. Adam SA, Geracet L (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. Cell 66(5):837–847
243. Kalderon D et al (1984) A short amino acid sequence able to specify nuclear location. Cell 39(3):499–509
244. Arenal A et al (2004) The SV40 T antigen nuclear localization sequence enhances nuclear import of vector DNA in embryos of a crustacean (Litopenaeus schmitti). Gene 337:71–77
245. Lim RY (2007) Gate-crashing the nuclear pore complex. Structure 15(8):889–891
246. Branden LJ, Mohamed AJ, Smith CI (1999) A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. Nat Biotechnol 17(8):784–787
247. Tanimoto M et al (2003) No enhancement of nuclear entry by direct conjugation of a nuclear localization signal peptide to linearized DNA. Bioconjug Chem 14(6):1197–1202
248. Neves C et al (1999) Intracellular fate and nuclear targeting of plasmid DNA. Cell Biol Toxicol 15(3):193–202

249. Sebestyen MG et al (1998) DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. Nat Biotechnol 16(1):80–85
250. Akita H et al (2006) Evaluation of the nuclear delivery and intra-nuclear transcription of plasmid DNA condensed with μ (mu) and NLS-μ by cytoplasmic and nuclear microinjection: a comparative study with poly-L-lysine. J Gene Med 8(2):198–206
251. Neves C et al (1999) Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation. FEBS Lett 453:41–45
252. Nagasaki T et al (2003) Can nuclear localization signals enhance nuclear localization of plasmid DNA? Bioconjug Chem 14(2):282–286
253. Zanta MA, Belguise-Valladier P, Behr JP (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proc Natl Acad Sci 96(1):91–96
254. Jeon O et al (2007) Poly(L-lactide-co-glycolide) nanospheres conjugated with a nuclear localization signal for delivery of plasmid DNA. J Drug Target 15(3):190–198
255. Jensen KD et al (2003) Cytoplasmic delivery and nuclear targeting of synthetic macromolecules. J Control Release 87(1–3):89–105
256. Burgess DJ, Kapoor M (2010) Quantum dot labeling for assessment of intracellular trafficking of therapeutically active molecules. In: Weissig V, D'Souza GGM (eds) Organelle-specific pharmaceutical nanotechnology. Wiley, New York, NY, pp 535–568
257. Chen F, Gerion D (2004) Fluorescent CdSe/ZnS nanocrystal-peptide conjugates for long-term, nontoxic imaging and nuclear targeting in living cells. Nano Lett 4(10):1827–1832
258. Lam AP, Dean DA (2010) Progress and prospects: nuclear import of nonviral vectors. Gene Ther 17(4):439–447
259. Merkwirth C, Langer T (2009) Prohibitin function within mitochondria: essential roles for cell proliferation and cristae morphogenesis. Biochim Biophys Acta, Mol Cell Res 1793(1): 27–32
260. Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15(22): 2922–2933
261. Chen M et al (2012) Chitosan/siRNA nanoparticles encapsulated in PLGA nanofibers for siRNA delivery. ACS Nano 6(6):4835–4844
262. Xun M-M et al (2013) Low molecular weight PEI-based biodegradable lipopolymers as gene delivery vectors. Org Biomol Chem 11(7):1242–1250
263. Rui M et al (2013) Recombinant high density lipoprotein nanoparticles for target-specific delivery of siRNA. Pharm Res 30(5):1203–1214
264. Y-y L et al (2013) Biocompatible polyethylenimine-graft-dextran catiomer for highly efficient gene delivery assisted with nuclear targeting ligand. Polym Chem 4:2528–2539
265. Du J et al (2012) Biodegradable nanoparticles of mPEG-PLGA-PLL triblock copolymers as novel non-viral vectors for improving siRNA delivery and gene silencing. Int J Mol Sci 13(1):516–533
266. Oishi M, Kataoka K, Nagasaki Y (2006) pH-responsive three-layered PEGylated polyplex micelle based on a lactosylated ABC triblock copolymer as a targetable and endosome-disruptive nonviral gene vector. Bioconjug Chem 17(3):677–688
267. Chu M et al (2013) Biocompatible polyethylenimine-graft-dextran catiomer for highly efficient gene delivery assisted by a nuclear targeting ligand. Polym Chem 4(8):2528–2539
268. Diez S, Navarro G, de Ilarduya CT (2009) In vivo targeted gene delivery by cationic nanoparticles for treatment of hepatocellular carcinoma. J Gene Med 11(1):38–45
269. Kim WJ et al (2006) Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. Mol Ther 14(3): 343–350
270. Kircheis R et al (2002) Tumor-targeted gene delivery: an attractive strategy to use highly active effector molecules in cancer treatment. Gene Ther 9(11):731–735

271. Lee M et al (2007) DNA delivery to the mitochondria sites using mitochondrial leader peptide conjugated polyethylenimine. J Drug Target 15(2):115–122
272. Matschke J et al (2012) Characterization of Ku702-NLS as bipartite nuclear localization sequence for non-viral gene delivery. PLoS One 7(2):e24615
273. McNamara JO et al (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 24(8):1005–1015
274. Schiffelers RM et al (2004) Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res 32(19):e149
275. de Tros Ilarduya C et al (2002) Enhanced gene delivery in vitro and in vivo by improved transferrin-lipoplexes. Biochim Biophys Acta 1561(2):209–221
276. Weissig V, De'Souza GGM, Torchilin VP (2001) DQAsome/DNA complexes release DNA upon contact with isolated mouse liver mitochondria. J Control Release 75(3):401–408
277. Wolfrum C et al (2007) Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat Biotechnol 25(10):1149–1157
278. Wolschek MF et al (2002) Specific systemic nonviral gene delivery to human hepatocellular carcinoma xenografts in SCID mice. Hepatology 36(5):1106–1114

Chapter 9
Mucosal Vaccine Delivery and M Cell Targeting

Prem N. Gupta

Abbreviations

APC	Antigen-presenting cells
BSM	Bovine submaxillary mucin
DODPC	1, 2-bis[(2E,4E)-Octadecadienoyl]-sn-glycero-3-phosphocholine
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
HBsAg	Hepatitis B surface antigen
HRP	Horseradish peroxidase
mAb	Monoclonal antibodies
NALT	Nasal associated lymphoid tissue
PLGA	Poly(D,L-lactide-co-glycolide)
PVA	Polyvinyl alcohol
sIgA	Secretory IgA
Tg	Glass transition temperature
UEA-1	*Ulex europaeus* agglutinin 1

9.1 Mucosal Immune System

The first productive interaction between most infectious agents and the host is with mucosal surfaces, specially, the nasal, oropharyngeal, respiratory, genitourinary, and gastrointestinal mucosa. Conventional vaccine strategies that involve parenteral

P.N. Gupta (✉)
Formulation and Drug Delivery Division, CSIR-Indian Institute of Integrative Medicine, Jammu, India
e-mail: pngupta@iiim.ac.in; pngupta10@gmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_9

immunization with inactivated viruses or bacteria or subunits of relevant virulence determinants of those pathogens do not prevent initial interactions. In-fact, traditional vaccine strategies do not prevent infection but instead resolve infection before disease ensues. Moreover, many bacterial toxins bind to and interact with mucosal epithelial cells, in which case significant damage to the host may ensue before serum antibodies can play a role in protection. The mucosal surfaces of the gastrointestinal and respiratory tracts represent the main portals of entry for most human pathogens. Sexual contact is another mucosal mode of transmission of infection. Direct inoculation of pathogens into the bloodstream is other important route of infection. Most external mucosal surfaces are replete with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B cells, T lymphocytes, T-cell subsets, plasma cells, and a variety of other cellular elements involved in the induction and maintenance of immune response. The mucosal surfaces encompass a critical component of the mammalian immunologic repertoire.

Numerous studies have indicated that induction of systemic immunity through parenteral immunization can effectively clear systemic infections, but it usually fails to protect the mucosal surfaces. Mucosal vaccine administration with an appropriate adjuvant, on the other hand, can induce immune responses at both systemic and mucosal sites and as a result, may prevent not only infectious diseases but also colonization at mucosal surfaces [1]. The mucosal immune system differs in several ways from the systemic immune system. Mucosal immunization frequently results in the stimulation of both mucosal and systemic immune responses, while systemic immunization typically only induces systemic responses without activating the mucosal immune system. Induction of mucosal response leads to production of secretory IgA (sIgA) antibodies, which are not usually produced by systemic immunization [2]. The production of sIgA on the mucosal surfaces is result of the local exposure of antigens to the mucosal-associated lymphoid tissues, especially those in the upper respiratory tract, and the gastrointestinal tract. In most cases infectious agents enter the body at mucosal surfaces and therefore the protective immunity at these surfaces can be effectively induced by mucosal immunization through oral, nasal, rectal, or vaginal routes [3].

The stimulation of the mucosal immune system at one mucosal site can lead to sIgA production in the local as well as distal mucosal surfaces. For example, antigen stimulation of the Peyer's patches in the gastrointestinal tract produced sIgA-producing B cells not only in the intestine, but also in the bronchi as well as in the genitourinary tract. This interconnected mucosal system of sIgA induction and production is termed as common mucosal immune system [2, 4]. The immunologic network operating on external mucosal surfaces consists of gut-associated lymphoid tissue (GALT), the lymphoid structures associated with bronchoepithelium and lower respiratory tract (BALT), ocular tissue, upper airway, salivary glands, tonsils, and nasopharynx (nasal associated lymphoid tissue; NALT), larynx (LALT), middle ear cavity, male and female genital tracts, and mammary glands. The organized lymphoid follicles in the GALT and BALT are main inductive sites of mucosal immune response.

9.2 Mucosal Immunization: An Edge Over Parenteral Vaccination

Vaccine delivery via mucosal route has several advantages over parenteral vaccination [5]. The most important reason for using a mucosal route of vaccination instead of a parenteral route is that the vast majority of infections occurs at or takes their departure from mucosal surfaces and in these infections mucosal vaccines are usually required to induce a protective immune response. The parenteral immunization induces poor mucosal immunity; however, mucosal immunization can induce both mucosal and systemic immunity [6]. The immunization at one mucosal site can induce specific responses at distant sites because of the expression of mucosa-specific homing receptors (site-specific integrins) by mucosally primed lymphocytes and complementary mucosal-tissue specific receptors (addressins) on the vascular endothelial cells [7]. This interconnected network is important because protective immunity (for instance against sexually transmitted diseases) could be induced in segregated mucosal sites in a practical way such as by oral or intranasal immunization and without hampering cultural or religious barriers. Mucosal vaccines are potentially useful to overcome the known barrier of parenteral vaccination caused by either preexisting systemic immunity from previous vaccination or in young children from maternal antibodies or selective immunosuppression such as that caused by HIV infection. For example, mucosal antibody response to oral cholera vaccination was observed in AIDS patients even after they had completely lost their ability to respond to an injectable vaccine (tetanus toxoid) [8]. In addition to serum IgG and mucosal IgA antibodies, mucosal immunization can stimulate cell mediated responses including helper CD4+ T cells and CD8+ cytotoxic T lymphocytes, the latter being important to eliminate intracellular pathogens [9]. The mucosal vaccine delivery is crucial for protective efficacy against noninvasive infections at mucosal surfaces that are normally impermeable to serum antibodies transduction, or passive passage across an epithelium, e.g., GIT infection with V. cholerae [7]. The mucosal vaccine delivery is particularly important for pathogens that can infect the host through both systemic and mucosal route because induction of both sIgA and systemic IgG confer protection at both site. This mode of vaccine delivery could be explored for combating pathogens acquired through non-mucosal routes such as blood or skin. Mucosal vaccination is also beneficial to induce peripheral systemic tolerance especially against those T cell mediated immune reaction that are associated with development of delayed type hypersensitivity reactions. This strategy is important to avoid delayed type hypersensitivity reactions and other allergic reactions to many ingested food proteins and other allergens [6]. Mucosal tolerance is a specific systemic hyporesponsiveness that arises after mucosal administration of an antigen. The tolerance is mediated by a combination of suppressor T-cells, inhibitory cytokines and factors which inhibit the inflammatory process. Oral tolerance can be used for the treatment of atopic diseases in human [10]. Oral tolerance varies when the antigen is administered in a soluble form as compared to a particulate state.

However, one major limitation with mucosal immunization is a striking balance between achieving an effective therapeutic response with a particular dose and preventing the induction of tolerance. Therefore, it is important to understand the mechanisms involved in controlling these responses. The key cells that determine the success or demise of a vaccine are antigen-presenting cells (APC), e.g., dendritic cells and T lymphocytes, including sub-populations of T-helper cells, T-cytotoxic cells, and regulatory T cells [7]. Immune interactions at the local level will have a profound effects on the type of immune response generated (e.g., first nuclear factor of activated T cell proteins is involved in both the generation of Th1 or Th2 cells and the maintenance of T-cell tolerance), so a clear understanding of local immune responses at the site of antigen uptake is essential [11].

On the other hand mucosal administration of vaccines also offers a number of practical advantages. Mucosal vaccination, being noninvasive in nature, does not require the use of needles. This would carry less risk of transmitting type of infections still associated with needle reuse [12, 13]. Additionally, mucosal vaccination is relatively easy and does not require expensive trained personnel. The production of mucosal vaccines may be cost effective in comparison to injectable vaccines that require high standards of purity, in addition to sterility. Moreover oral vaccines can also be expected to have much greater acceptability than injectable vaccines by causing no sore arm etc. Further it can enhance vaccine safety and adverse effect by avoiding direct contact between potentially toxic vaccine component and systemic circulation. Finally, mucosal vaccines allow for the easy administration of multiple vaccines [14].

9.3 M Cell as Gateway of Mucosal Immune Systems

The common features of all inductive mucosal sites include epithelial surface containing M cells overlying organized lymphoid follicles (Fig. 9.1). M cells offer functional openings in the epithelial barrier through vesicular transport activity [15]. They are well suited for efficient endocytosis and transcytosis. The M cells lack the rigid brush border cytoskeleton of their enterocyte neighbors, and their apical surfaces have broad membrane, a microdomain from which endocytosis occurs. The M cell basolateral membrane is deeply invaginated to form a large intraepithelial "pocket" containing T lymphocytes, B lymphocytes, and macrophages. This structural specialization brings the basolateral cell surface to within a few microns of the apical surface and greatly shortens the distance that transcytotic vesicles must travel to cross the epithelial barrier. Endocytic or phagocytic uptake of foreign antigens or particles is followed by rapid transcytosis directly to the intraepithelial pocket, with little or no retention in M cell lysosomes. After M cell transport, antigens are processed and presented by macrophages, dendritic cells, and B cells within and below the epithelium, resulting in generation of IgA-committed, antigen-specific B lymphoblast that proliferate locally in the germinal centers of O-MALT and migrate via the bloodstream to distant mucosal and glandular tissues, where

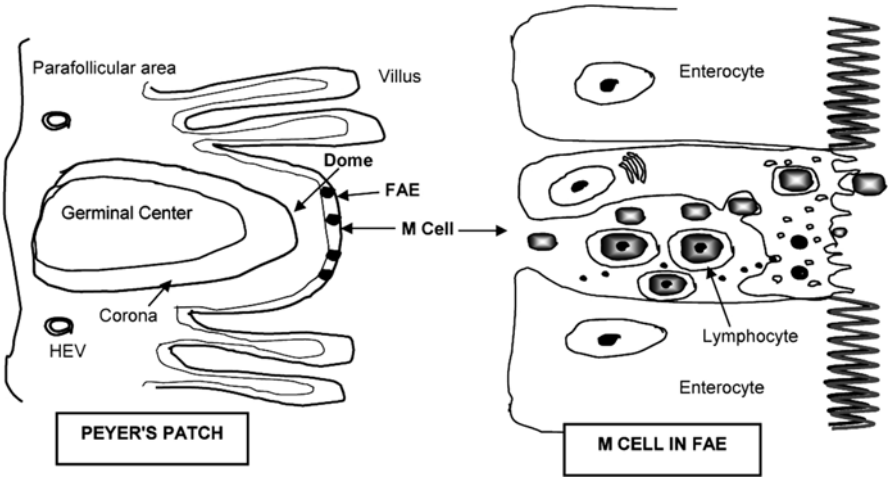

Fig. 9.1 M cells of the Peyer's patches

they differentiate into plasma cells. The dimeric or polymeric IgA antibodies thus produced are selectively bound by epithelial polymeric immunoglobulin receptors, transcytosed across epithelial cells, and released into glandular and mucosal secretions [16]. Thus, M cell plays a pivotal role in the elicitation of secretory immune response.

9.4 Distinguishing Feature of M Cells

The M cell apical surfaces are distinguished from their enterocyte neighbors by the absence of a typical brush border and the presence of variable microvilli [17] and a unique intraepithelial "pocket." This hallmark structural feature of fully differentiated M cells, provides a docking site for lymphocytes and shortens the distance that transcytotic vesicles must travel from the apical to the basolateral surface. The basolateral surface of the M cell includes the two major subdomains typical of all epithelial cells: the lateral subdomain is involved in cell–cell adhesion and contains Na+-K+-ATPase, and the basal subdomain interacts with the extracellular matrix and basal lamina.

The M cell apical surface differs from that of intestinal absorptive cells in other respects as well. Most M cells in Peyer's patches lack the highly organized brush border with uniform, closely packed microvilli typical of enterocytes. The actin-associated protein villin, confined to microvilli in enterocytes, is diffusely distributed in M cells [18], reflecting the modified apical organization and perhaps the ability to rapidly respond to adherence of microorganisms with ruffling and phagocytosis. M cells are also recognized by their lack of certain enterocytes surface glycoproteins. Whereas enterocyte brush borders have abundant hydrolytic enzymes,

these enzymes are usually reduced or absent on M cells [19, 20]. In addition, the thick filamentous glycocalyx typical of enterocytes is often absent from M cells, rendering the M cell surface more accessible to luminal materials. Further, M cell apical surfaces are coated with glycoproteins that display glycosylation patterns different from their enterocyte neighbors, and although the protein backbones have not been identified, the carbohydrate epitopes can be useful M cell identifiers which can be explored for targeted vaccine delivery.

9.5 M-Cell Surface Receptors

On the basis of the observation that M cells are targeted by a variety of soluble tracers and inert particles, it has been suggested that M cell targeting by microorganisms may be mediated, at least in part, by nonspecific, passive mechanisms. Such mechanisms are likely to be influenced by the physical properties of the pathogen, since the interaction of inert particles with M cells is influenced by the physicochemical properties of the particle preparation and by species related variations in M cell surface properties [21–24]. Additionally, specific receptors located in either the M cell apical membranes or the closely associated glycocalyx also contribute to M cell targeting by microorganisms. In the view of the fact that M cells bind and endocytose antibodies located in the lumen, there is interest in ascertaining whether there are specific Ig receptors on M cells and the follicle associated epithelium (FAE). A novel IgA receptor was discovered in mouse M cells with the potential to facilitate transport of sIgA from luminal secretions into GALT [25]. The finding that sialylated Lewis A antigen (SLAA) appeared to be expressed selectively on M cells from a small number of biopsies of human Peyer's and cecal patches was very exciting [26]. Although a "universal" M-cell marker is lacking, some exciting results from targeting experiments have been observed. For example, the coupling of recombinant cholera toxin B subunit to liposomes containing *Streptococcus mutans* antigens generated enhanced mucosal immunization in mice compared to untargeted antigen-loaded particles [27].

Ulex europaeus agglutinin 1 (UEA-1) has high specificity for the carbohydrate moiety, α-L-fucose, located on the apical membranes of mouse M cells [28, 29]. There have been successful efforts made into in vivo targeting to mouse M cells by conjugating the lectin to polymerized liposomes [30] and also to latex particles [31]. There are many success stories of UEA-1based delivery system is animal models; however, it is of limited value in vaccine delivery in human because the lectin is toxic, is subject to intestinal degradation, and its receptor is not expressed in human PP [26]. As another alternative to the lectin UEA-1, the edible orange peel mushroom *Aleuria aurantia* was used to target the α-L-fucose receptor. Coated poly(D,L-lactide-co-glycolide) (PLGA) particles were entrapped with birch pollen antigens and administered to mice as a potential oral allergen immunotherapy [32]. M cells can also be discriminated from enterocytes in the FAE on the basis of altered adherens junction protein expression [33]. Various other receptors and markers on FAE or M cells from various species have been summarized by Brayden et al. 2005 [34].

9.6 Mucosal Vaccine Delivery Systems

The mucosal vaccine delivery systems can be classified into live vectors (e.g., *Salmonella typhi*) and nonliving antigen delivery systems (Liposomes, nanoparticles, immunostimulating complexes, etc.). These mucosal vaccine carriers are summarized in Table 9.1, and in this chapter two potential mucosal vaccine delivery systems, i.e., polymeric particles and liposomes are described.

Table 9.1 Various options for the vaccine delivery by mucosal route

Option for mucosal vaccine delivery	Comments	Ref.
Live bacterial vector, (*Salmonella, E. coli., Mycobacterium*)	The capability of some microorganism to colonize and infect intestinal mucosa and the potential for including genes for unrelated microorganism encoding relevant antigens represent an attractive means for design of novel mucosal vaccines	[35, 36]
Live viral vector, (Vaccinia virus, Canary pox virus, Picornaviruses)	Live recombinant vector vaccines have the advantage that they can stimulate both humoral and cell mediated immune responses and have potential for immunization alone or in combination with a subunit vaccine	[37]
Virosomes	Viral surface glycoproteins possess high affinity for receptors on mucosal surfaces thus providing a mechanism for efficient attachment of antigen to mucosal surfaces	[38]
Liposomes	Liposome vaccine may enhance uptake and processing by enclosing the antigen in the lipid vesicles. Although they are not completely resistant to lipases and bile salts found in the small intestine, cholesterol-containing liposomes can provide at least partial resistance. Polymerized liposomes are considered to be a good candidate for the oral immunization	[30]
Nanoparticles and microparticles	Particles can be taken up by the M cells of the Peyer's patches. Nanoparticles/microparticles have advantage over microbial system in which immune response to the live vector can dominate	[39, 40]
Cochleates	Induce a strong and prolonged immune response manifested by the presence of mucosal and systemic antibody and cytotoxic T cells	[41]
Mucoadhesive polymers	Mucoadhesive polymer avoids the complexity of microencapsulation technology. They have been tested for nasal immunization but had been overlooked for oral vaccine delivery	[42]
Cholera toxin B subunit conjugates	Proteins coupled to CTB acquire its mucosal immunogenic properties due to the high affinity of CTB for cell surface G_{M1} ganglioside and its avid uptake by M cells on intestinal Peyer's patches	[43, 44]
Immune-stimulating complex matrix (ISCOM)	ISCOMs are cage like structures into which antigen can be incorporated resulting in enhanced immune response after their administration. ISCOMs are resistant to solubilization by the bile salts deoxycholate, cholate, and taurocholate	[45]
DNA delivery to mucosal surface	Direct mucosal administration of DNA plasmid expression vector encoding a protein antigen is more efficient than recombinant viral vector for gene transfer to muscle tissue. Mannosylated niosomes are also demonstrated to be a potential adjuvant carrier for oral genetic immunization	[46]
Transgenic plants	This technology represent an important step for the production of inexpensive edible immunogen suitable for immunization of large population	[47, 48]

9.6.1 Polymeric Particles

It has been demonstrated that the incorporation of an antigen into microparticles [49], or its adsorption to the surface of biodegradable microparticles [50], resulted in the induction of enhanced serum and secretory antibody responses, following oral administration. Subsequently, it was shown that oral delivery of an antigen entrapped in PLGA microparticles also resulted in the induction of enhanced immunity [51]. During last two decades biodegradable PLGA based microparticles/nanoparticles have been explored extensively [52–54]. Binding and uptake of the particles was enhanced when particles were conjugated to B subunit of *E. coli* heat labile enterotoxin (LTB), the plant lectin, ConA or vitamin B12 following oral delivery to rats [55]. Covalent attachment of UEA-1 to polystyrene microspheres and oral delivery to mice result in selective binding to and rapid uptake by the Peyer's patch M-cells [56]. Orally administered polystyrene microparticles with attached *Lycopersicon esculentum* agglutinin (LEA) were taken to a greater extent than unconjugated particles in the rats [57]. The linkage of sepharose beads to WGA and *Solanum tuberosum* lectin (STL) enhanced their binding to caco-2 cells [58]. These observations and a body of additional data suggest that lectins are potential tools for the enhanced binding and internalization of orally delivered drugs and drug delivery systems and efficiency of oral vaccines can be improved by M-cell targeting.

We have described the development of PLGA nanoparticles loaded with HBsAg and the antigen stabilization in the presence of trehalose and $Mg(OH)_2$. Additionally, UEA-1 lectin was anchored to the nanoparticles to target them to M-cells of the Peyer's patches [59]. The results suggest that HBsAg can be successfully stabilized by co-encapsulation of protein stabilizers. The lectinized nanoparticles have demonstrated approximately fourfold increase in the degree of interaction with the bovine submaxillary mucin (BSM) as compared to plain nanoparticles and sugar specificity of the lectinized nanoparticles was also maintained. The serum anti-HBsAg titre obtained after oral immunization with Hepatitis B surface antigen (HBsAg) loaded stabilized lectinized nanoparticles was comparable with the titre recorded after alum-HBsAg given intramuscularly. The stabilized UEA-1 coupled nanoparticles exhibited enhanced immune response as compared to stabilized non-lectinized nanoparticles. Furthermore, the stabilized lectinized nanoparticles elicited sIgA in the mucosal secretion and IL-2 and IFN-γ in the spleen homogenates.

9.6.1.1 Factors Affecting Uptake of Polymeric Particles

The M cell appears to be the primary route of entry into the host for several enteric viral pathogens. The mechanism for the uptake of synthetic and biodegradable microparticles by M cells appears similar to that observed for bacteria. Microparticle uptake initially involves contact with the microvillus projections on the M cell

surface followed by rapid phagocytosis through the extension of apical membrane processes. Various factors affecting the uptake of particles are described below.

Particle Size

In general, smaller microparticles are absorbed to a greater degree than larger microparticles. The smaller particles are distributed more easily to distant sites, and remain detectable for longer periods of time. These conclusions are consistent for microparticles made of different polymers and different size ranges. Jani et al. [60] studied the comparative uptake of 50 nm, 500 nm, and 1 μm polystyrene particles and found that 50 nm particles are absorbed and distributed quicker than 500 nm and 1 μm particles. Florence suggested that decrease in particle diameter may result in increased uptake below 1 μm and particles above 3 μm are taken up by the Peyer's patches but remained there [61].

Hydrophobicity

Hydrophobicity of the particles influences profoundly their uptake behavior. Jung et al. reported that uptake of nanoparticles prepared from hydrophobic polymer was higher than from particles with more hydrophilic surfaces [62]. They further added that hydrophobic polystyrene nanoparticles interact with M cells with more affinity than absorptive epithelia whereas less hydrophobic PLGA nanoparticles interact with both cell types. Other investigators have shown that decreasing surface hydrophobicity, by the adsorption of poloxamers 235, 238, 407, or poloxamines 901, 904, and 908, may decrease the uptake of polystyrene microparticles into cells of the immune system, thereby avoiding elimination [63]. The charge on the particles also determines their uptake by the intestinal epithelia. Although the charged particles are taken up, their uptake was less than the non-ionic hydrophobic particles [61]. The negatively charged and neutral particles exhibited greater affinity to PP in comparison to positively charged particles [64]. This finding was in accordance with previous report that a combination of both, negative charge and increased hydrophobicity of the particles improve the gastrointestinal uptake [62].

Effect of Dose and Vehicle on Uptake

The extent of particle uptake is also influence by the dosing. It was observed that polystyrene particles were identified in Peyer's patches with difficulty after 1 day of feeding, but were readily identified following chronic feeding. Le Ray et al. [65] have shown that changing the vehicle in which the particles were administered could enhance the extent of uptake of polystyrene particles in mice. Further, volume and tonicity of the administered vehicle also have an effect on the extent of uptake [66].

Glass Transition Temperature and Crystallinity of the Polymer

Glass transition temperature (Tg) and crystallinity of the polymer are two important bulk properties of the polymer affecting the release of incorporated components. Without proper release characteristics, drugs or vaccines incorporated into microparticles may be released either prematurely or to insignificant levels before elimination. Tg is the temperature at which a transition occurs from the glassy state to the rubbery state resulting in increase in the molecular motion and free volume of the amorphous polymer, which in turn increases the drug release from the polymer. Above Tg the polymer acquires sufficient thermal energy for isomeric rotational motion or for significant torsional oscillation to occur about most of the bonds in the main chain. This leads to an increase in the free volume of the amorphous polymer, and thus in turn, the release of incorporated bioactive [67].

Migliaresi et al. observed an increase in the degree of crystallinity of polylactic acid with the degradation of the polymer [68]. This could be related to the faster degradation of the amorphous phase of the semicrystalline polymer, resulting in loss of amorphous material and a concomitant increase in crystallinity. A decrease in crystallinity increases the drug release because the diffusion coefficient and solubility of the drug in polymer are inversely proportional to at least the first power of the amorphous content. The structural features, which influence the crystallinity of the polymer, are similar to those, which affect the glass transition temperature.

Bioactive (drug/vaccine) may release from nanoparticles/microparticles by several mechanisms including surface and bulk erosion, disintegration, microparticle hydration, drug diffusion and desorption. These bioactive release mechanisms are in turn controlled by bulk properties such as the molecular weight of the polymer (affecting crystallinity and glass transition temperature), the copolymer composition, polymer matrix density and the extent and nature of the cross-linking. By adjusting the blend ratio of PLGA/polyethyleneglycol (PEG) the release profile of entrapped dextran and rabbit gamma immunoglobulin (IgG) microparticles can be varied [69]. The release rate of entrapped compounds increased with increasing PEG content because of the leaching out of PEG from the polymer blend into the aqueous phase during drug release, resulting in the increased porosity of microparticles. Changing the monomer ratio of lactide/glycolide in PLGA microparticles from 75:25 to 50:50 led to an increase in the release rate of entrapped OVA due to an increase in the degradation rate of the microparticles [70].

Effect of Additives

Various additives are involved in the fabrication of nanoparticles/microparticles. Polyvinyl alcohol (PVA) is the most commonly used emulsifier in the formulation of lactide and poly (D,L-lactide-co-glycolide) nanoparticles/microparticles. A fraction of PVA remains associated with the nanoparticles/microparticles despite repeated washing because PVA forms an interconnected network with the polymer at the interface. The residual PVA affect different pharmaceutical properties of the

particles such as particle size, zeta potential, polydispersity index, surface hydrophobicity, protein loading and also slightly influenced the in-vitro release of encapsulated protein. Importantly, nanoparticles with higher amount of residual PVA had relatively lower cellular uptake despite their smaller particle size [71]. The lower cellular uptake of nanoparticles with higher amount of residual PVA is attributed to the higher hydrophilicity of the nanoparticle surface. Trehalose is well-documented protein stabilizer. We have observed increase in the release of HBsAg with trehalose stabilized PLGA nanoparticles/microparticles when compared with PLGA nanoparticles/microparticles without trehalose [72]. Since the protein stabilizer (trehalose) reduced denaturation at the aqueous-organic interface, the payload of HBsAg was increased and this was reflected in augmented cumulative percent release. Moreover, sugars (e.g., trehalose, sucrose) have appreciable solubility in aqueous media. They dissolve rapidly from the matrix leaving a porous matrix, which in turn releases antigen/bioactive relatively faster.

Effect of Species, Animal Age, and Food Ingestion on Uptake

The species variation can affect uptake extent of the particles. The uptake of polystyrene particles in rabbit was at least an order of magnitude greater than mice because of the greater abundance of the M cells in the Peyer's patches [73]. Le Fevre et al. showed greater uptake of polystyrene particles in older mice [74]. Other investigator reported that age of the animal did not affect the extent of polystyrene particle uptake in rats [75]. The extent of uptake in the mice was enhanced by the presence of food, which may delay the intestinal transit of the particles [75].

Intestinal Mucus Layer Characteristics

The uptake of the nanoparticles/microparticles is preceded by their passage through two barriers, i.e., the mucus gel layer and the mucosa. Intestinal mucus is a high molecular weight glycoprotein secretion, which covers the mucosa with a continuous adherent blanket. The mucus layer protects the gastrointestinal mucosa from potentially harmful bacteria, pathogens, or chemicals [76]. Several investigators have reported diminished diffusion of small and large compounds such as bovine serum albumin (BSA), lysozyme, tertiary amines, and quaternary ammonium compounds [77]. Mucus acts as a barrier by entrapping microparticles, causing agglomeration, which results in an increase in net size and a resultant decrease in diffusion coefficient, and by decreasing the diffusion coefficient through the mucus thereby restricting diffusion to the mucosa layer. Since the high number of sulfate, sialic acid, and sugar moieties in the carbohydrate side chains of the mucin molecule impart a highly negative charge to mucin [76], it may be expected that electrostatic interactions between positively charged drugs and particles would cause binding within the mucin layer. Several mechanisms have been documented in literature for the uptake of the nanoparticles/microparticles (Table 9.2).

Table 9.2 Site-specific mechanism for the uptake of the nanoparticles/microparticles

Uptake site	Mechanism	Particle size
Intestinal epithelial cells on villus tip	Paracellular transport	100–200 nm
Villus tips	Persorption	5–150 μm
Intestinal macrophages	Phagocytosis	1 μm
Enterocytes/M cells	Endocytosis	<200 nm
Peyer's patches	Transparacellular	<10 μm

9.6.2 Liposomes

Although some success has been achieved in experimental studies involving oral administration of antigens entrapped in liposomes [78], disappointing results have also been reported by others [79, 80]. Nevertheless, a liposomal vaccine has been shown to induce a salivary IgA response in a small number of human volunteers, following oral immunization [81]. Furthermore, although it has been reported that liposomes are unstable in the gut and are not taken up by epithelial cells [82], the uptake of liposomes into Peyer's patches has been reported [83]. Polymerized liposomes exhibited many characteristics which make them attractive antigen carriers. They are in the nanometer size range suitable to be transported by M cells. Further, liposomes formed by polymerization of 1, 2-bis[(2E,4E)-Octadecadienoyl]-sn-glycero-3-phosphocholine (DODPC) can be manipulated by incorporation of different lipid groups, thus adjusting surface charge and rigidity. DODPC liposomes also have carboxylate groups on their surface which could facilitate cell receptor targeting [84]. A number of studies showed the potential of targeted liposomes in the induction of immune response. Sugimoto et al. [85] and Fukasawa et al. [86] showed that liposomes coated with mannopentose and dipalmitoyl phosphatidylethanolamine (Man5-DPPE) could elicit strong cellular immune responses. It has been shown that UEA1-coated liposomes can be efficiently targeted to murine M cells in vivo [30], thus supporting the hypothesis that decorating liposomes with M cell specific lectins may efficiently target orally delivered antigens to M cells and possibly DCs residing in the FAE.

Liposome offers a number of potential advantages for the mucosal vaccine delivery [87]. While native liposomes may target Peyer's patch M-cells [83], the efficiency of binding and subsequent uptake is thought to be relatively low following oral gavage of mice [88, 89]. Therefore, we have developed lectin conjugated liposomes for M-cell targeted vaccine delivery [90]. The activity of the liposome-conjugated with UEA-1 towards exogenously provided BSM and affinity toward competing sugar were studied to determine targeting efficacy of lectinized liposomes. The lectinized liposomes showed good BSM binding in absence of specific sugar for UEA-1 (α-L-fucose). The same formulations, however, showed significant decrease in the percent BSM binding in the presence of α-L-fucose (Fig. 9.2). M-cell targeting of the liposomes was studied by dual staining by using CLSM. The interaction of liposomes with M-cells may be facilitated by the relatively thin M-cell

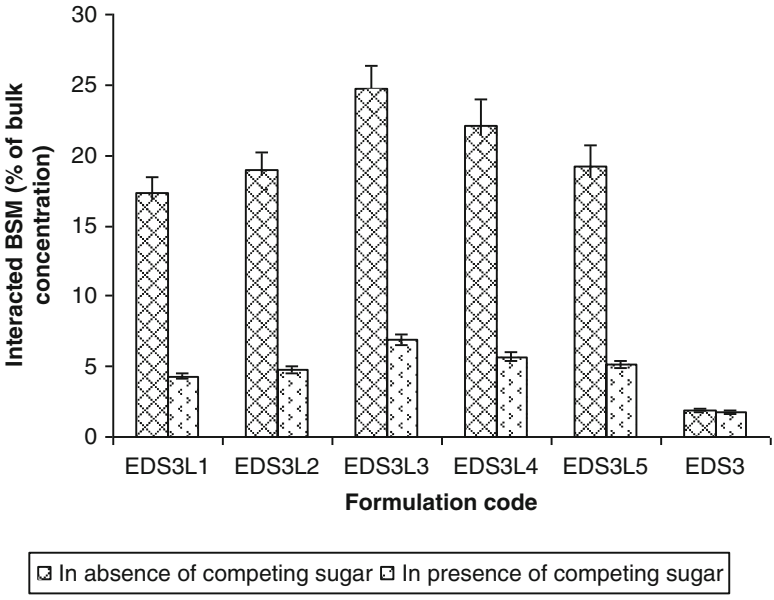

Fig. 9.2 Binding of BSM to lectinized liposomes (EDS3L1-EDS3L5) and optimized non-lectinized liposomes (EDS3) in presence and absence of competing sugar (Reproduced with permission from [90])

glycocalyx which appears to promote the interaction of small particles with the M-cell surface membranes. Also, there are various other factors which may affect the Peyer's patch uptake of particles [5]. Lectinized liposomes showed higher immune response in comparison to non-lectinized formulation (Figs. 9.3 and 9.4). UEA-1 anchored liposomes selectively targeted to M-cells of Peyer's patch, and M-cell adherent liposomes are thought to be rapidly endocytosed. M-cell apical surfaces are coated with glycoproteins that display glycosylation patterns different from their enterocyte neighbors, and although the protein backbones have not been identified, the carbohydrate epitopes can be useful M-cell identifiers [91]. Thus, M-cell targeted liposomes were found to have greater accessibility to M-cell and as a consequence they showed enhanced immune response as compared to non-lectinized liposomes.

9.7 Targeting of Mucosal Vaccine Delivery Systems

Targeting to the specific site of the gastrointestinal tract is an effective means for enhancing the uptake of the particulate systems. Depending on the pharmaceutical application, different targets within the gastrointestinal tract can be exploited (Fig. 9.5)

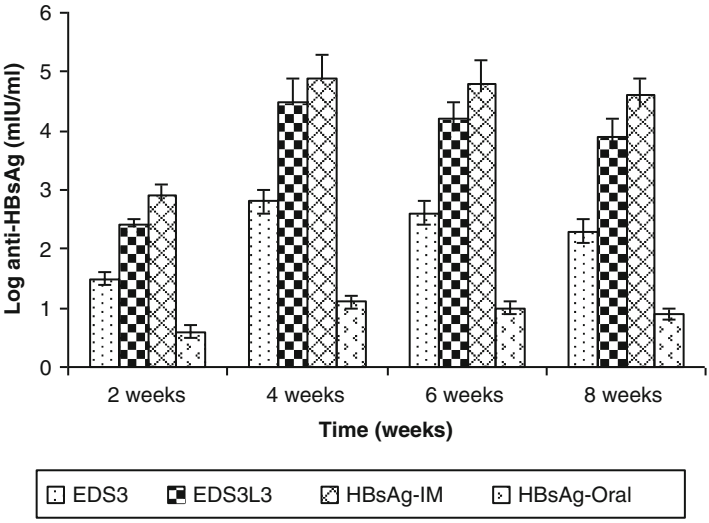

Fig. 9.3 Serum anti-HBsAg antibody levels with lectinized liposomal (EDS3L3) and optimized non-lectinized liposomes (EDS3). The animals were immunized orally with the preparation equivalent to the 10 μg HBsAg for three consecutive days and booster dose was given after third week of first immunization. Single intramuscular immunization with booster dose after third week was given with the alum-HBsAg to serve as standard and HBsAg without liposomes was administered orally followed by a booster dose after third week for the comparison. After 4 weeks there was significant difference ($P<0.001$) among EDS3L3, EDS3, HBsAg-Oral or HBsAg-IM, however, the difference between EDS3L3 and HBsAg-IM was not significant ($P>0.05$). (Reproduced with permission from [90])

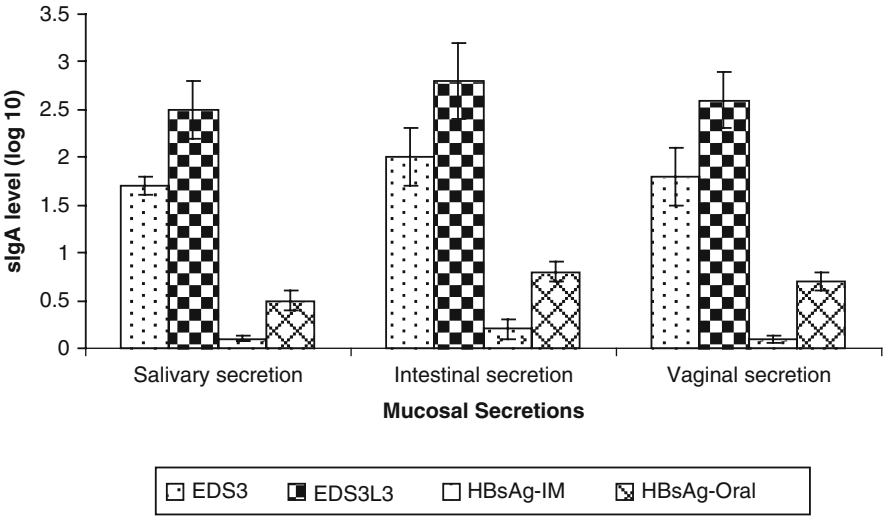

Fig. 9.4 sIgA level in mucosal secretions after oral immunization with HBsAg encapsulating lectinized liposomes (EDS3L3) and optimized non-lectinized liposomes (EDS3). The animals were immunized orally with the preparation equivalent to the 10 μg HBsAg for 3 consecutive days and booster dose was given after third week of first immunization. Single intramuscular immunization with booster dose after third week was given with the alum-HBsAg to serve as standard and HBsAg without liposomes was administered orally followed by a booster dose after third week for the comparison. The salivary, intestinal and vaginal secretions were collected after eight week of first immunization. The differences in the antibody level was significant ($P<0.05$) among EDS3L3, EDS3, HBsAg-Oral or HBsAg-IM. (Reproduced with permission from [90])

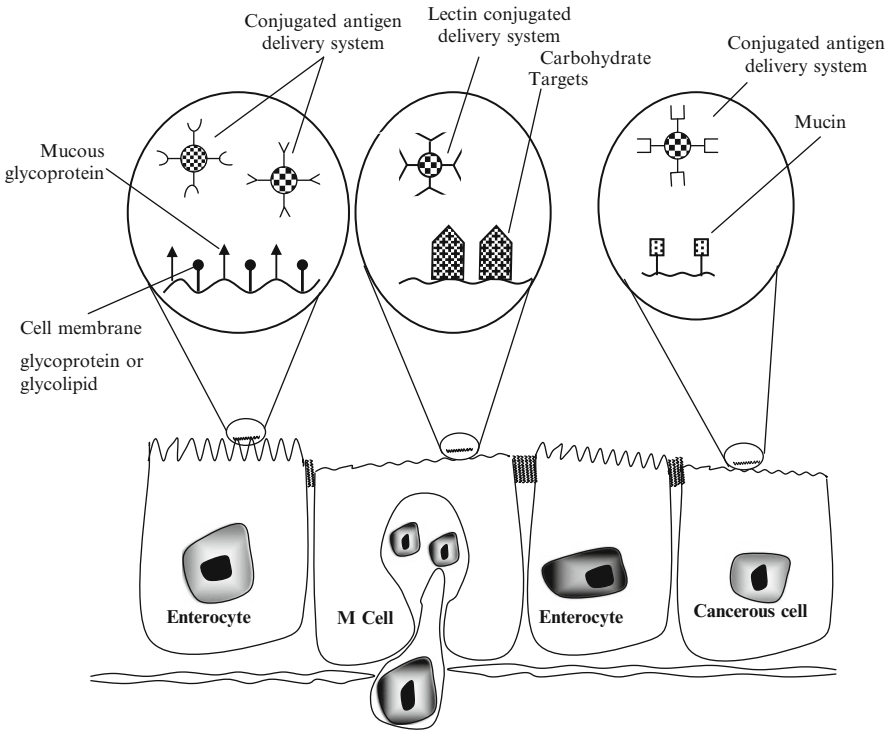

Fig. 9.5 Various options for the targeted delivery. Mucus glycoprotein, M-cells and abnormal glycoprotein secreted by the cancerous cells can serve as receptor for binding with various ligands conjugated to drug/vaccine delivery system

including mucus glycoproteins (mucins), epithelial cells, M-cells, Peyer's patches or GALT, and abnormal glycoproteins secreted by cancerous cells (local tumors). Brayden et al. reviewed novel M-cell surface receptors that could be used to target orally delivered antigens [34]. Gene expression technology has provided evidence that coculture model has many characteristics of Peyer's patches. It has been demonstrated that epithelial genes that were unregulated in coculture corresponds to genes expressed selectively in mouse FAE [92]. These include claudin 4, laminin β3, tetraspanTM4SF3 and a matrix metalloproteinase. Claudin 4 appears to have dual location at tight junctions (M cell–enterocyte), and as an M cell and enterocyte cytoplasmic receptor, it is involved in the trafficking of pathogens across M cells to lymphocyte or dendritic cells. Peptidoglycan recognition protein (PGRP)-S and PGRP-L are other potential targets co-localized with UEA-positive cells in microdissected mouse Peyer's patches and in the FAE respectively [93]. Other targeting agents like lectins, invasins, antibodies, etc. can be used as a means of enhancing targeting and thus in turn particle uptake. Various ligands for the targeted immunization are summarized in Table 9.3.

Table 9.3 Various ligands for targeted mucosal immunization

Targeting ligand	Targeting site	Conjugated material	Ref.
Ulex europaeus 1	Mouse Peyer's patch M cells	FITC, HRP	[94]
		Polystyrene microparticles	[56]
		Liposomes	[89]
mAb 5B11	Brush border of both M cells	Polystyrene latex microparticles	[95]
Lycopersicon esculentum	Rat intestine	Polystyrene microparticles	[96]
Secretory IgA	Mouse Peyer's patch M cells	Polystyrene microparticles	[97]
		Liposomes	[98]
Triticum vulgaris	Mouse intestine	Liposomes	[89]
Bandeiraea simplicifolia I isolectin B 4	Hamster NALT	Biotin, HRP	[99]
	M cells		
Invasin-C192	Intestinal M cells	Polystyrene nanoparticles	[100]
O-palmitoyl mannan	Peyer's patch	Niosomes	[101]
Cholera toxin B subunit	M cells of Peyer's patches	Liposomes	[102]
		Bilosomes	[103]

9.7.1 Lectin Mediated Targeting

Lectins are proteins or glycoproteins capable of specific recognition of and reversible binding to carbohydrate determinants of complex glycoconjugates, without altering the covalent structure of any of the recognized glycosyl ligands. Lectin receptors are expressed on various cells such as endothelial cells, hepatocytes, macrophages, monocytes and lymphocytes. They are efficient in recognizing the complex oligosaccharide epitopes, which are also present on the cell surface or could be exogenous glycoconjugate ligands mimics of endogenous carbohydrate epitopes [104]. Lectins are potential tools for the targeting of particulate vaccines to the M cell of the Peyer's patches, which are the sampling site of the mucosal immune system. Nanoparticles/microparticles may also be targeted to mouse Peyer's patch M cells by coating with the lectin UEA1 for the development of an effective mucosally targeted vaccines. In studies reported by Foster et al. [56], polystyrene microparticles (0.5-mm diameter) were covalently coated with the lectin UEA1 and administered to mice both by injection into ligated gut loops of anaesthetized animals and by oral gavage. In contrast to other proteins, lectin UEA1 coating selectively targeted the microparticles to mouse Peyer's patch M cells, and M cell adherent microparticles were rapidly endocytosed. Although the lectins specific for the human intestinal M cells await identification, human M cells preferentially display the sialyl Lewis A antigen [26] and this could be envisaged for targeting vaccines to the mucosal immune system. Future studies should determine whether lectins may similarly be used to target vaccine candidate in PLA/PLGA based delivery construct to intestinal M cells, and whether such targeting enhances the immune response to antigens delivered by these carrier systems. Recently our group has developed biodegradable polymer based stabilized microparticles and nanoparticles for the mucosal vaccination [39] and also envisaged lectin for the targeted

mucosal immunization [40]. Additionally we have also explored cholera toxin B subunit conjugated bilosomes [43] and mannosylated niosomes [101] as potential carrier-adjuvants for the targeted oral mucosal immunization.

9.7.2 Invasin Mediated Uptake

Invasins are virulent factor usually associated with the bacterial cell wall, and have the capacity to stimulate cytokine synthesis and to interact with mammalian cells by distinct mechanisms [105]. Young et al. demonstrate the potential of invasins for the internalization process [106]. For this purpose, microparticles were coated with Yersinia enterocolitica invasin and the resulting conjugates put in contact with human laryngeal epithelial cells (Hep-2 cells). The presence or absence of internalised conjugates was monitored by transmission electron microscopy and light microscopy. It was clear that conjugates not only bound, but also were internalised by the Hep-2 cells. In contrast, control conjugates were rarely associated with these cells. *Salmonella typhimurium* selectively bind to, invade and destroy murine M cells and have been studied as live oral vaccine delivery vehicles. The M cell targeting by *S. typhimurium* is mediated by a specific adhesin (long polar fimbria; LPF) [107]. Reovirus type 1 is another ligand, which selectively adhere to, and endocytosed by intestinal M cells. It was demonstrated that proteolytic processing of native reovirus type 1 is required for adhesion to murin M cells and this is dependent on retention of modified σ1 and/or product of μ1 outer capsid protein. It has been suggested that σ1 protein has potential for targeted delivery [108].

9.7.3 Antibody Directed Targeting

The use of antibodies and monoclonal antibodies has been proposed for specific targeting within the gastrointestinal tract. It was observed that binding of the 5B11 monoclonal antibody, with specificity for rabbit M cells, to polystyrene particles, enhanced uptake by rabbit M-cells 3–3.5-times when compared to controls (plain latex and IgM of unrelated specificity-conjugates) [109]. The ability of different conjugates, obtained by coating latex microparticles with albumin, bovine growth hormone (bGH), human IgG, secretory IgA (hIgA), and bGH complexed with an IgG antibody raised against bGH (bGH-Ab), to be taken up by M cells was studied. It was found that the selectivity in binding to and entry into M-cells was improved by the use of IgG or bGH-Ab. Moreover, the appearance of conjugates in rat mesenteric lymph showed a similar selectivity to that found for binding and entry into M-cells. Ferritin-loaded liposomes conjugated to IgA were investigated for mucosal immunization via the rectum [109]. It was observed that the presence of IgA on the liposome surface increased the uptake of conjugates by Peyer's patches, and the local rectal/colon immune response to ferritin about fivefold over uncoated liposomes.

9.8 Link Between M Cell Uptake of Particles and Induction of Mucosal Immunity

Generally, macromolecules that adhere to mucosal surfaces tend to induce vigorous mucosal immune responses, whereas soluble, non-adherent proteins do not [110]. Pathogens and vaccines that can bind selectively to M cells appear to be most effective in mucosal invasion and induction of mucosal immune responses, and this assumption underlies many of the current approaches to vaccine design. Presently, there have been few successful oral vaccine trials in man using non-live antigens in particles. Mixed results were obtained in a limited number of human subjects dosed with untargeted PLG microspheres containing the highly potent *E. coli* colonization factor antigen II as potential vaccine for enterotoxigenic *E. coli* [111]. These studies suggest indirectly that there is likely to be some particle uptake in man; however, a quantifiable relationship between enhanced M-cell targeting of vaccine loaded particles and an enhanced immune outcome remains illusive. Apart from the immunology issues, pharmaceutical factors are critical in the design of antigen-loaded particles. These include antigen stability issues, premature antigen release from particles in the intestine and incomplete antigen release within Peyer's patch at the right time. There are many reports of induction of immune response using M cell targeted particles in animal models; however, more clinical studies are required to establish a correlation between M cell targeting and elicitation of immune response. A Phase I trial of a single shot tetanus toxoid (TT) and diphtheria toxoid in PLGA microspheres is still some way off, even though outcomes in mice and guinea-pigs showed positive and durable immune responses using antigen-loaded microparticles [54]. It is possible that particulate antigens, when targeted to inductive immune sites, might perform even better in man than in laboratory animals because the pathways of antigen presentation by human dendritic cells are relatively well established and might even be superior [112]. Advancement in the non-living vaccine delivery system coupled with the suitable targeting strategy may lead to a successful clinical trial.

9.9 Future Perspectives

Recent discoveries in both mucosal vaccine delivery and mucosal adjuvant research have significantly improved the effectiveness of mucosal immunization in animal models. The mucosal immune system is a complex system that generate large amount of s-IgA as well as cell mediated immunity at mucosal surfaces to prevent pathogen infiltration and inflammation. The mucosal immune system should be most efficient in providing protection against pathogens and generating longer lasting protection through using attenuated pathogen for vaccine. The only mucosal vaccines approved for humans are attenuated pathogens. Future mucosal vaccines will also involve vaccines strategies other than attenuated pathogens. New delivery strategies such as immunization of live recombinant vectors, DNA plasmids, and

transgenic plants to deliver antigens present promises to improve the efficiency of mucosal antigen delivery. Further, DNA vaccines and subunit vaccines such as bacterial adhesion in combination with potent mucosal adjuvants (such as QS21; a saponin, unmethylated CpG motifs or cytokines such as IL-12) or mucosal delivery systems based on nanoparticles/microparticles will have the potential to be the next generation of vaccines.

Mucosal delivery of vaccines offers a number of significant advantages over systemic delivery. There are many alternative approaches to the mucosal delivery of vaccines and our group has explored various versions of delivery systems [39, 40, 43, 90, 101, 113]. One potential approach to the mucosal delivery of vaccines is the encapsulation or entrapment of antigens into polymer based nanoparticles or microparticles. Polymeric delivery systems can be manipulated to enhance the efficacy of mucosally administered vaccines in a number of ways; they can protect antigens from degradation, concentrate them in one area of the mucosal tissue for better absorption, extend their residence time in the body, or target them to sites of antigen uptake (e.g., Peyer's patches in the gut).

Immunization does not always stimulate immunity because of the insufficient elicitation of immune responses. Such limitations have spurred the development of new adjuvant and antigen-delivery systems. Adjuvant plays an important role in enhancing the efficacy of vaccines. Recombinant proteins or synthetic peptides are safer than crude inactivated microorganism, but less immunogenic. This limitation can be overcome by using specific adjuvant. The adjuvant selection depends on several criteria, like the target species, the antigens, the type of desired immune response, the route of administration, or the duration of immunity. So far, biodegradable polymers particularly of PLGA have been used, considerably because of their well-known degradation properties. An area requiring additional efforts is analytical characterization of protein-encapsulated nanoparticles/microparticles. Advanced methods for protein characterization is in demand to approach problem of protein stabilization in polymer based delivery systems. Development of in vitro–in vivo correlation for protein release from protein nanoparticles/microparticles is another issue. More intensive interactions between immunologists and drug delivery specialists are required to understand protein release and its presentation to the immune system. Significant progress has been made recently with biodegradable polymer, mainly PLGA and various approaches are being considered for the effective stabilization of proteins in microparticles during the preparation process. Among them we have focused on the basic additives mediated stabilization of therapeutic protein within carrier construct [39, 40]. Nevertheless, all the approaches involving encapsulation of antigens into nanoparticles/microparticles are likely to suffer from the some significant drawback; the extent of uptake of the particles across the gut appears to be limited. Whether or not the extent of uptake in humans is sufficient to allow the development of an effective oral vaccine is currently unknown. However, it is clear that in rodents, the extent of uptake of nanoparticles/microparticles can be enhanced using targeting ligands.

Numerous studies suggest that the efficiency of particle absorption can be improved through modification of particle surfaces with targeting molecules such as

antibodies or lectins. Albeit the results are promising, it is not known if any of the strategies will be effective in humans because it is currently unclear if particulates are taken up in human GIT. Additionally the uptake mechanisms and absorption efficiencies are not known. Thus, current knowledge obtained from animal models may or may not be extendible to human beings. Continued research to understand the interconnection and sub-compartmentalization of the common mucosal system will certainly guide the rational selection for routes of mucosal administration. An efficient delivery vehicle, combined with an effective adjuvant given through an optimal route of administration, will ultimately allow for the development of a successful needle-free (mucosal) vaccine in humans.

References

1. Davis SS (2001) Nasal vaccines. Adv Drug Deliv Rev 51:21–42
2. Nugent J, Po AL, Scott EM (1998) Design and delivery of non-parenteral vaccines. J Clin Pharm Ther 23:257–285
3. Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6:148–158
4. Mestecky J, Michalek SM, Moldoveanu Z, Russell MW (1997) Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. Behring Inst Mitt 98:33–43
5. Vyas SP, Gupta PN (2007) Implication of nanoparticles/microparticles in mucosal vaccine delivery. Expert Rev Vaccines 6:401–418
6. Holmgren J, Czerkinsky C, Eriksson K, Mharandi A (2003) Mucosal immunization and adjuvants: a brief overview of recent advances and challenges. Vaccine 21:S2/89–S2/95
7. Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11(Suppl 4): S45–S53
8. Eriksson K, Kilander A, Hagberg L, Norkrans G, Holmgren J, Czerkinsky C (1998) Induction and expression of intestinal homoral immunity in HIV-infected individuals: prospects for vaccination against secondary enteric infections. Pathobiology 66:176–182
9. Magistris MTD (2006) Mucosal delivery of vaccine antigens and its advantages in pediatrics. Adv Drug Deliv Rev 58:52–67
10. Ogra PL, Faden H, Welliver RC (2001) Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 14:430–445
11. Macian F (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5:472–484
12. Levine MM (2003) Can needle-free administration of vaccines becomes the norm in global immunization? Nat Med 9:99–103
13. Ryan EJ, Daly LM, Mills KHG (2001) Immunomodulators and delivery systems for vaccination by mucosal routes. Trends Biotechnol 19:293–304
14. Chen H (2000) Recent advances in mucosal vaccine development. J Control Release 67:117–128
15. Neutra MR, Pringault E, Kraehenbuhl JP (1996) Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14:275–300
16. Apodaca G, Bomsel M, Arden J, Breitfeld PP, Tang KC, Mostov KE (1991) The polymeric immunoglobulin receptor: A model protein to study transcytosis. J Clin Invest 87: 1877–1882
17. Owen RL (1977) Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology 72:440–451

18. Kerneis S, Bogdanova A, Colucci-Guyon E, Kraehenbuhl JP, Pringault E (1996) Cytosolic distribution of villin in M cells from mouse Peyer's patches correlates with the absence of a brush border. Gastroenterology 110:515–521
19. Owen RL, Bhalla DK (1983) Cytochemical analysis of alkaline phosphatase and esterase activities and of lectinbinding and anionic sites in rat and mouse Peyer's patch M cells. Am J Anat 168:199–212
20. Savidge TC, Smith MW (1995) Evidence that membranous (M) cell genesis is immunoregulated. In: Mestecky J (ed) Advances in mucosal immunology. Plenum, New York
21. Jepson MA, Clark MA, Foster N, Mason CM, Bennett MK, Simmons NL, Hirst BH (1996) Targeting to intestinal M cells. J Anat 189:507–516
22. Jepson MA, Simmons NL, O'Hagan DT, Hirst BH (1993) Comparison of poly(DL-lactide-co-glycolide) and polystyrene microsphere targeting to intestinal M cells. J Drug Target 1:245–249
23. Ermak TH, Giannasca PJ (1998) Microparticle targeting to M cells. Adv Drug Deliv Rev 34:261–283
24. Clark MA, Jepson MA, Hirst BH (2001) Exploiting M cells for drug and vaccine delivery. Adv Drug Deliv Rev 50:81–106
25. Mantis NJ, Cheung MC, Chintalacharuvu KR, Rey J, Corthesy B, Neutra MR (2002) Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. J Immunol 169:1844–1851
26. Giannasca PJ, Giannasca KT, Leichtner AM, Neutra MR (1999) Human intestinal M cells display the sialyl Lewis A antigen. Infect Immun 67:946–953
27. Harokopakis E, Hajishengallis G, Michalek SM (1998) Effectiveness of liposomes possessing surface-linked recombinant B subunit of cholera toxin as an oral antigen delivery system. Infect Immun 66:4299–4304
28. Clark MA, Jepson MA, Simmons NL, Booth TA, Hirst BH (1993) Differential expression of lectin-binding sites defines mouse intestinal M-cells. J Histochem Cytochem 41:1679–1687
29. Giannasca PJ, Giannasca KT, Falk P, Gordon JI, Neutra MR (1994) Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. Am J Physiol 267:G1108–G1121
30. Clark MA, Blair H, Liang L, Brey RN, Brayden D (2001) Targeting polymerized liposome vaccine carriers to intestinal M cells. Vaccine 20:208–217
31. Lambkin I, Pinilla C, Hamashin C, Spindler L, Russell S, Schink A, Moya-Castro R, Allicotti G, Higgins L, Smith M, Dee J, Wilson C, Houghten R, O'Mahony D (2003) Toward targeted oral vaccine delivery systems: selection of lectin mimetics from combinatorial libraries. Pharm Res 20:1258–1266
32. Roth-Walter F, Scholl I, Untersmayr E, Fuchs R, Boltz-Nitulescu G, Weissenbock A, Scheiner O, Gabor F, Jensen-Jarolim E (2004) M cell targeting with Aleuria aurantia lectin as a novel approach for oral allergen immunotherapy. J Allergy Clin Immunol 114:1362–1368
33. Clark MA, Hirst BH (2002) Expression of junction-associated proteins differentiates mouse intestinal M cells from enterocytes. Histochem Cell Biol 118:137–147
34. Brayden DJ, Jepson MA, Baird AW (2005) Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today 10:1145–1157
35. Banerjee S, Medina-Fatimi A, Nichols R (2002) Safety and efficacy of low-dose Escherichia coli enterotoxin adjuvant for urease-based oral immunization against Helicobacter pylori in healthy volunteers. Gut 51:634–640
36. Metzger WG, Mansouri E, Kronawitter M (2004) Impact of vector-priming on the immunogenicity of a live recombinant Salmonella enterica serovar typhi Ty21a vaccine expressing urease A and B from Helicobacter pylori in human volunteers. Vaccine 22:2273–2277
37. Smythies LE, Novak MJ, Waites KB, Lindsey JR, Morrow CD, Smith PD (2005) Poliovirus replicons encoding the B subunit of Helicobacter pylori urease protect mice against H. pylori infection. Vaccine 23:901–909
38. Durrer P, Gluck U, Spyr C (2003) Mucosal antibody response induced with a nasal virosome-based influenza vaccine. Vaccine 21:4328–4334

39. Jaganathan KS, Vyas SP (2006) Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant Hepatitis B antigen administered intranasally. Vaccine 24:4201–4211
40. Gupta PN, Mahor S, Rawat A, Khatri K, Goyal A, Vyas SP (2006) Lectin anchored stabilized biodegradable nanoparticles for oral immunization: development and in-vitro evaluation. Int J Pharm 318:163–173
41. Perez O, Bracho G, Lastre M (2006) Proteoliposome-derived Cochleate as an immunomodulator for nasal vaccine. Vaccine 24:S2/52–S2/53
42. Alpar HO, Somavarapu S, Atuah KN, Bramwell VW (2005) Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. Adv Drug Deliv Rev 57:411–430
43. Singh P, Prabakaran D, Jain S, Mishra V, Jaganathan KS, Vyas SP (2004) Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization. Int J Pharm 278:379–390
44. Kozlowski PA, Williams SB, Lynch RM (2002) Differential induction of mucosal and systemic antibody responses in women after nasal, rectal or vaginal immunization: influence of the menstrual cycle. J Immunol 169:566–574
45. Aguila A, Donachie AM, Peyre M, McSharry CP, Sesardic D, Mowat AM (2006) Induction of protective and mucosal immunity against diphtheria by an immune stimulating complex (ISCOMS) based vaccine. Vaccine 24:5201–5210
46. Jain S, Singh P, Mishra V, Vyas SP (2005) Mannosylated niosomes as adjuvant–carrier system for oral genetic immunization against Hepatitis B. Immunol Lett 101:41–49
47. Rice J, Ainlev WM, Shewen P (2005) Plant-made vaccines: biotechnology and immunology in animal health. Anim Health Res Rev 6:199–209
48. Thanavala Y, Mahoney M, Pal S (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci U S A 102:3378–3382
49. O'Hagan DT, Palin NK, Davis SS (1989) Poly(butyl-2-cyanoacrylate) particles as adjuvants for oral immunisation. Vaccine 7:213–216
50. O'Hagan DT, Palin NK, Davis SS, Artursson E, Sjoholm I (1989) Microparticles as potentially orally active immunological adjuvants. Vaccine 7:421–424
51. Eldridge JH, Hammond CJ, Meulbroek JA, Stass JK, Gilley RM, Tice TR (1990) Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches. J Control Release 11:205–214
52. Gutierro I, Hernandez RM, Igartua M, Gascon AR, Pedraz JL (2002) Size dependent immune response after subcutaneous, oraland intranasal administration of BSA loaded microspheres. Vaccine 21:67–77
53. Yeh MK, Liu YT, Chen JL, Chiang CH (2002) Oral immunogenicity of the inactivated Vibrio cholerae whole cell vaccine encapsulated in biodegradable microparticles. J Control Release 82:237–247
54. Katz DE, Delorimier AJ, Wolf MK, Hall ER, Cassels FJ, van Hamont JE, Newcomer RL, Davachi MA, Taylor DN, McQueen CE (2003) Oral immunization of adult volunteers with microencapsulated enterotoxigenic Escherichia coli (ETEC) C56 antigen. Vaccine 21:341–346
55. Russell-Jones GJ (2001) The potential of receptor mediated endocytosis for oral drug delivery. Adv Drug Deliv Rev 1:59–73
56. Foster N, Clark MA, Jepson MA, Hirst BH (1998) Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo. Vaccine 16:536–541
57. Florence AT, Hillery A, Hussain N, Jani PU (1995) Factors affecting the oral uptake and translocation of polystyrene nanoparticles: Histological and analytical evidence. J Drug Target 3:65–70
58. Gabor F, Stangl M, Wirth M (1995) Lectin mediated bioadhesion: binding characteristics of plant lectins on the enterocyte like cell lines Caco-2, HT-29 and HCT-8. J Control Release 55:131–142
59. Gupta PN, Khatri K, Goyal AK, Mishra N, Vyas SP (2007) M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. J Drug Target 15:701–713

60. Jani P, McCarthy DE, Florence AT (1992) Nanosphere and microsphere uptake via Peyer's patches: observation of the rate of uptake in the rat after a single oral dose. Int J Pharm 86:239–246
61. Florence AT (2005) Nanoparticle uptake by oral route: fulfilling its potential? Drug Discov Today Technol 2:75–81
62. Jung T, Kamm W, Breitenbach A, Kaiserling E, Xiao JX, Kissel T (2000) Biodegradable nanoparticles for oral delivery of peptides: is there is a role for polymers to affect mucosal uptake? Eur J Pharm Biopharm 50:147–160
63. Moghimi SM, Hawley AE, Christy NM, Gray T, Illum L, Davis SS (1994) Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes. FEBS Lett 344:25–30
64. Shakweh M, Besnard M, Nicolas V, Fattal E (2005) Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by Peyer's paches in mice. Eur J Pharm Biopharm 61:1–13
65. Le Ray AM, Vert M, Gautier JC, Benoit JP (1994) Fate of poly (DL-lactide-co-glycolide) nanoparticles after intravenous and oral administration to mice. Int J Pharm 106:201–211
66. Eyles J, Alpar HO, Field WN, Lewis DA, Keswick M (1995) The transfer of polystyrene microspheres from the gastrointestinal tract to the circulation after oral administration in the rat. J Pharm Pharmacol 47:561–565
67. Norris DA, Puri N, Sinko PJ (1998) The effect of physical barriers and properties on the oral absorption of particulates. Adv Drug Deliv Rev 34:135–154
68. Migliaresi C, Fambri L, Cohn D (1994) A study on the in-vitro degradation of poly (lactic acid). J Biomater Sci Polym Ed 5:591–606
69. Cleek RL, Ting KC, Eskin SG, Mikos AG (1997) Microparticles of poly (DL-lactic-co-glycolic acid)/poly (ethylene glycol) blends for controlled drug delivery. J Control Release 48:259–268
70. O'Hagan DT, Jeffery H, Davis SS (1994) The preparation and microcharacterization of poly (lactide-co-glycolide) microparticles: III microparticle/polymer degradation rates and the in vitro release of model proteins. Int J Pharm 103:37–45
71. Sahoo SK, Panyam J, Prabha S, Labhasetwar V (2002) Residual polyvinyl alcohol associated with poly (D, L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release 82:105–114
72. Jaganathan KS, Singh P, Prabakaran D, Mishra V, Vyas SP (2004) Development of a single-dose stabilized poly (D, L-lactic-co-glycolic acid) microsphere-based vaccine against hepatitis B. J Pharm Pharmacol 56:1243–1250
73. Pappo J, Ermak TH (1989) Uptake and translocation of fluorescent latex particle by rabbit Peyer's patch follicle epithalium: a quantitative model for M cell uptake. Clin Exp Immunol 76:227–280
74. Le Fever ME, Boccio AM, Jeol DD (1989) Intestinal uptake of fluorescent microsphere in young and aged mice. Proc Soc Exp Biol Med 190:23–27
75. Simon L, Shine G, Dayan AD (1994) Effect of animal age on the uptake of large particulates across the epithelium of rat small intestine. Int J Exp Pathol 75:369–373
76. Strous GJ, Dekker J (1992) Mucin-type glycoproteins. Crit Rev Biochem Mol Biol 27:57–92
77. Desai MA, Mutlu M, Vadgama P (1992) A study of macro-molecular diffusion through native porcine mucus. Experientia 48:22–26
78. Michalek SM, Childers NK, Katz J, Denys FR, Berry AK, Eldrigge JH, McGee JR, Curtiss R (1989) Liposomes as oral adjuvants. Curr Top Microbiol Immunol 146:51–58
79. Clarke CJ, Strokes CR (1992) The intestinal and serum humoral immune responses of mice to systemically and orally administered antigens in liposomes: I. The response to liposome entrapped soluble proteins. Vet lmmunol Immunopathol 32:125–138
80. Clarke CJ, Strokes CR (1992) The intestinal and serum humoral immune responses of mice to systemically and orally administered antigens in liposomes: II. The response to liposome entrapped bacterial proteins. Vet lmmunol Immunopathol 32:139–148

81. Childers NK, Michalek SM, Pritchagd DG, McGhee JR (1991) Mucosal and systemic responses to an oral liposome Streptococcus mutans carbohydrate vaccine in humans. Reg Immunol 3:289–296
82. Chiang CM, Weiner N (1987) Gastrointestinal uptake of liposomes. I In vitro and in situ studies. Int J Pharm 37:75–85
83. Childers NK, Denys FR, McGhee NF, Michalek SM (1990) Ultrastructural study of liposome uptake by M cells of rat Peyer's patches: an oral vaccine system for delivery of purified antigen. Reg Immunol 3:8–16
84. Foster N, Hirst BH (2005) Exploiting receptor biology for oral vaccination with biodegradable particulates. Adv Drug Deliv Rev 57:431–450
85. Sugimoto M, Ohishi K, Fukasawa M, Shikata M, Kawai H, Itakura H, Hatanaka M, Sakakibara R, Ishiguro M, Nakata M (1995) Oligomannose-coated liposomes as an adjuvant for the induction of cell-mediated immunity. FEBS Lett 363:53–56
86. Fukasawa M, Shimizu Y, Shikata K, Nakata M, Sakakibara R, Yamamoto N, Hatanaka M, Mizuochi T (1998) Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of CD8+ cytotoxic T lymphocytes. FEBS Lett 441:353–356
87. Childers NK, Michalek SM (1994) Liposomes. In: O'Hagan DT (ed) Novel delivery systems for oral vaccines. CRC, Boca Raton, FL
88. Chen H, Torchilin V, Langer R (1996) Polymerized liposomes as potential oral vaccine carriers: stability and bioavailability. J Control Release 42:263–272
89. Chen H, Torchilin V, Langer R (1996) Lectin-bearing polymerized liposomes as potential oral vaccine carriers. Pharm Res 13:1378–1383
90. Gupta PN, Vyas SP (2011) Investigation of lectinized liposomes as M-cell targeted carrier-adjuvant for mucosal immunization. Colloids Surf B Biointerfaces 82:118–125
91. Neuta MR, Mantis NJ, Frey A, Giannasca PJ (1999) The composition and function of M cell apical membranes: implications for microbial pathogenesis. Semin Immunol 11:171–181
92. Lo D, Tynan W, Dickerson J (2004) Cell culture modeling of specialized tissue: identification of genes expressed specifically by follicle associated epithelium of Peyer's patch by expression profiling of caco-2/Raji co-cultures. Int Immunol 16:91–99
93. Lo D, Tynan W, Dickerson J (2003) Peptidoglycan recognition protein expression in mouse Peyer's patch follicle associated epithelium suggests functional specialization. Cell Immunol 224:8–16
94. Clark MA, Jepson MA, Simmons NL, Hirst BH (1995) Selective binding and transcytosis of Ulex europaeus 1 lectin by mouse Peyer's patch M-cells in vivo. Cell Tissue Res 282:455–461
95. Kabok Z, Ermak TH, Pappo J (1994) Microheterogeneity of follicle epithelium and M cells in rabbit gut-associated lymphoid tissues defined by monoclonal antibodies. FASEB J 8: A1008
96. Carreno-Gomez B, Woodley JF, Florence AT (1999) Studies on the uptake of tomato lectin nanoparticles in everted gut sacs. Int J Pharm 183:7–11
97. Porta C, James PS, Phillips AD, Savidge TC, Smith MW, Cremaschi D (1992) Confocal analysis of fluorescent bead uptake by mouse Peyer's patch follicle-associated M cells. Exp Physiol 77:929–932
98. Zhou F, Kraehenbuhl JP, Neutra MR (1995) Mucosal IgA response to rectally administered antigen formulated in IgA-coated liposomes. Vaccine 13:637–644
99. Giannasca PJ, Boden JA, Monath TP (1997) Targeted delivery of antigen to hamster nasal lymphoid tissue with M-cell- directed lectins. Infect Immun 65:4288–4298
100. Hussain N, Florence AT (1998) Utilizing bacterial mechanisms of epithelial cell entry: Invasin-induced oral uptake of latex nanoparticles. Pharm Res 15:153–156
101. Jain S, Vyas SP (2006) Mannosylated niosomes as adjuvant-carrier system for oral mucosal immunization. J Liposome Res 16:331–345
102. Harokopakis E, Childers NK, Michalek SM, Zhang SS, Tomasi M (1995) Conjugation of cholera toxin or its B subunit to liposomes for targeted delivery of antigens. J Immunol Methods 185:31–42

103. Conacher M, Alexander J, Brewer JM (2001) Oral immunization with peptide and protein antigens by formulations in lipid vesicles incorporating bile salts (bilosomes). Vaccine 19:2965–2974
104. Vyas SP, Singh A, Sihorkar V (2001) Ligand-receptor-mediated drug delivery: an emerging paradigm in cellular drug targeting. Crit Rev Ther Drug Carrier Syst 18:1–76
105. Henderson B, Poole S, Wilson M (1996) Bacterial modulins: novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol Rev 60:316–341
106. Young VB, Falkow S, Schoolnik GK (1992) The invasin proteinof Yersinia enterocolitica: internalization of invasin bearing bacteria by eukaryotic cells is associated with reorganization of the cytoskeleton. J Cell Biol 116:197–207
107. Jepson MA, Clark MA (1998) Studying M cells and their role in infection. Trends Microbiol 6:359–365
108. Clark MA, Hirst BH, Jepson MA (2000) Lectin-mediated mucosal delivery of drugs and microparticles. Adv Drug Deliv Rev 43:207–223
109. Durrer C, Irache JM, Duchene D, Ponchel G (1994) Study of the interactions between nanoparticles and intestinal mucosal. Prog Colloid Polym Sci 97:275–280
110. De Aizpurua HJ, Russell-Jones GJ (1988) Oral vaccination: identification of classes of proteins that provoke an immune response upon oral feeding. J Exp Med 167:440–451
111. Tacket CO, Reid RH, Boedeker EC, Losonsky G, Nataro JP, Bhagat H, Edelman R (1994) Enteral immunization and challenge of volunteers given enterotoxigenic E. coli CFA/II encapsulated in biodegradable microspheres. Vaccine 12:1270–1274
112. Peyre M, Sesardic D, Merkle HP, Gander B, Johansen P (2003) An experimental divalent vaccine based on biodegradable microspheres induces protective immunity against tetanus and diphtheria. J Pharm Sci 92:957–966
113. Jain S, Sharma RK, Vyas SP (2006) Chitosan nanoparticles encapsulated vesicular systems for oral immunization: preparation, in-vitro and in-vivo characterization. J Pharm Pharmacol 58:303–310

Part V
Physicochemical Approaches for Targeting

Chapter 10
Stimuli Responsive Carriers: Magnetically, Thermally and pH Assisted Drug Delivery

Eameema Muntimadugu, Anjali Jain, and Wahid Khan

Abbreviations

ABC	ATP-binding cassette
DEA	Diethylacrylamide
DOPE	Dioleoylphosphatidylethanolamine
ECM	Extracellular matrix
LCST	Lower critical solution temperature
NIPAM	*N*-isopropylacrylamide
PCLA	Poly(ε-caprolactone-co-lactide)
PAD	Poly(*N*-amidino)dodecyl acrylamide
PEO	Polyethylene oxide
PNIPAM–CS	Poly(*N*-isopropylacrylamide)–chitosan
NVCL	*N*-vinylcaprolactam
UCST	Upper critical solution temperature

10.1 Introduction

Clinical application of most of the drugs is limited by their side effects in spite of their beneficial action. There has been a long time desire to achieve selective delivery of bioactives to target areas in the body in order to maximize therapeutic potential and minimize side-effects. For achieving better therapeutic application,

E. Muntimadugu • A. Jain • W. Khan (✉)
Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, India
e-mail: wahid@niperhyd.ac.in

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_10

nanocarriers are considered for target-specific delivery of drugs to various sites in the body in order to improve the therapeutic efficacy, while minimizing undesirable side effects [1]. Nanocarriers possess in vivo longevity and specific capability of extravasation through the endothelium of inflammatory tissues (the so-called enhanced permeability and retention effect), whereas their functionalisation with biologically active ligands facilitates the targeting of specific cells. However, the translation of both the enhanced permeability and retention effect and ligand recognition into the clinic still remains questionable. This may be, to a certain extent a consequence of the stochastic nature of ligand–receptor interactions and of difficulties in the control of the release of the drug from targeting nanocarriers. One alternative involves on-demand processes (also termed "switch on/off"), which in principle allow for tailored release profiles with excellent spatial, temporal and dosage control. On-demand drug delivery is becoming feasible through the design of stimuli responsive systems that recognize their microenvironment and react in a dynamic way, mimicking the responsiveness of living organisms [2]. The concept of stimuli-responsive drug delivery was first suggested in the late 1970s with the use of thermosensitive liposomes for the local release of drugs through hyperthermia. Since then, and particularly in the past decade a great deal of research has been carried out on stimuli-responsive materials for drug delivery, especially concerning their design and applications as nanocarriers [3].

Stimuli-responsive nanocarriers are specialised nano-sized active delivery vehicles that evolve with an external signal and are equipped with "load-and-release" modalities within their constituting units. The central operating principle of these carriers lies in the fact that a specific cellular/extracellular stimulus of chemical, biochemical, or physical origin can modify the structural composition/conformation of the nanocarriers, thereby promoting release of the active species to specific biological environment. The observed changes are mainly decomposition, isomerisation, polymerisation, activation of supramolecular aggregation among many others. The general concept of triggered release can be divided mainly into two major modes according to the nature of the interaction between the bioactive molecule and the nanocarriers. In the complexation approach, where the bioactive agent is entrapped within the nanocarrier, the release can be triggered by structural change within the carrier scaffold (i.e. carrier degradation, cleavage of shell, charging of functional groups), while in the nanocarrier-conjugate approach; the mechanism of release involves the splitting of the linker between the carrier and the bioactive agent. The external stimuli which bring about these changes are numerous and cross related. These advanced nanocarriers thus become an active participant in the therapeutic landscape, rather than an inert carrier molecule [4].

10.2 Classification

Stimuli that trigger drug release from the nanocarriers can be broadly classified with respect to the biological systems as either endogenous (physiological, pathological, and patho-chemical conditions) or exogenous (physical) (Fig. 10.1).

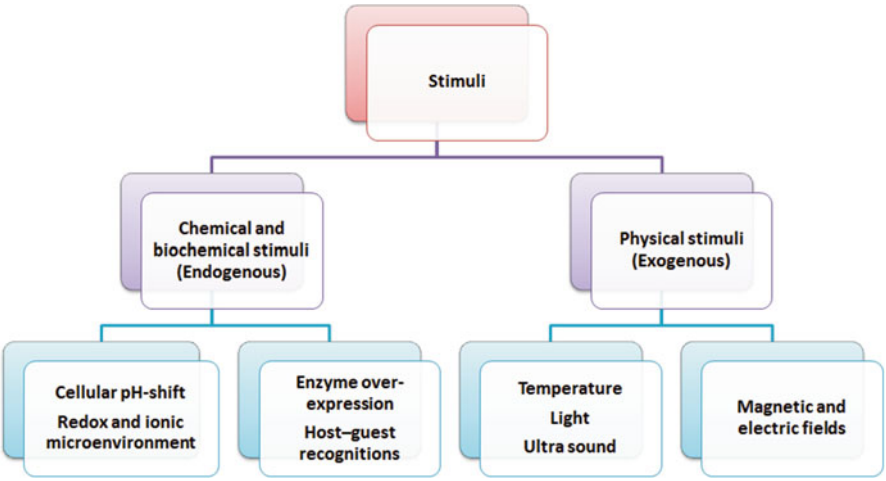

Fig. 10.1 Classification of stimuli involved in responsive drug delivery systems

Endogenous stimuli of chemical and biochemical origin include cellular pH-shift, redox, and ionic microenvironment of the specific tissues, enzyme over-expression in certain pathological states, host–guest recognitions, and antigen–antibody interactions. Physical stimuli that can be applied externally to bring about a triggered release of active guest may involve temperature, light, mechanical pressure, and strength of magnetic or electrical fields. This chapter throws light on pH, thermally and magnetic field assisted drug delivery either alone or as dual responsive systems.

10.3 pH-Responsive Carriers

Among the common stimuli, pH-responsiveness is the most frequently used. These carriers respond to pH gradients within the microenvironments of organs, tissues and cell organelles to achieve drug release at desired site. Certain tumours and inflamed tissue have a slightly lower pH values (between pH 5.4 and 7.4) than homeostatic conditions (pH 7.4). Furthermore, there exists a lower intracellular pH in endosomes and lysosomes. As such particles internalised through endocytosis will experience a pH gradient from neutral (pH ~ 7.4) in extracellular environment, to acidic (pH ~ 6.2) in early endosome and more acidic (pH ~ 5.0) in lysosome [5, 6]. Moreover, members of the ATP-binding cassette (ABC) efflux pump superfamily, such as P-glycoprotein (P-gp)/ABCB1, MDR-associated protein/ABCC, and breast cancer resistance protein/ABCG2, play important roles in drug kinetics including absorption, distribution, metabolism and excretion, which limits the accumulation of drugs inside the cells and results in drug resistance [7]. pH responsive carriers are expected to provide fast intracellular drug release and make the intracellular drug

concentration to reach a sufficiently high level to exceed the efflux capacity of drug transporters and the threshold concentration to kill the MDR tumour cells.

There are several general approaches of such systems that undergo chemical transitions around the critical pH range of 5–7 [8]. One approach is to introduce "titratable" or "protonizable" chemical groups such as amines and carboxylic acids into the components assembling the nanocarriers. The systems containing amines or carboxylic groups with different chemical structures and pKa values could change their physical and chemical properties, such as swelling ratio or solubility in response to local pH level [9]. Another approach is to incorporate acid-labile linkages directly to attach drugs covalently to the vectors or into the main-chains of the polymers constructing the carriers. The pH sensitive bonds are cleaved at acidic pH, accompanied by dissolution of carriers and release of drugs. The third approach is to incorporate carbon dioxide (CO_2)-generating ingredient for inducing CO_2 gas in acidic environment and leading to disintegration of the vehicles [10].

10.3.1 Delivery Systems

pH-responsive systems are mainly designed and reported as dendrimer, liposomes, nanoparticles and nanofibres (Fig. 10.2 and Table 10.1). Dendrimers are highly branched oligomers or polymers characterised by three structural features: (1) the central core from which the polymeric branches emanate, (2) the nature of the repeating unit which determines the microenvironment of the interior and thus the solubilisation ability of the dendrimer and (3) the nature and number of the terminal functional groups, mainly responsible for the behavior of dendrimers in solution. Pistolis et al. Developed pyrene loaded poly(propyleneimine) dendrimers for pH dependent release of pyrene. The release was increased up to tenfold by decreasing the pH to 2–4 [11]. Dual acting pH and thermosensitive dendrimer with a shell of poly(*N*,*N*-dimethylaminoethyl methacrylate) were also developed [12]. Similarly Yuan et al., reported pH-sensitiveness and cellular targeting dendrimer to provide the advantage of thermo regulated targeting. This system contained poly(L-glutamic acid) dendrimers with a polyhedral oligomeric silsesquioxane nanocubic core. Doxorubicin was attached via pH-sensitive hydrazine bonds and biotin was used as targeting moiety [13].

As another delivery system, pH-sensitive liposomes are designed to undergo acid-triggered destabilisation. For this, first generation pH-sensitive liposomes which are based on the cone-shaped lipid dioleoylphosphatidylethanolamine and later, serum-resistant pH-sensitive liposome formulations containing egg phosphatidylcholine and cholesteryl hemisuccinate are developed. These liposomes exhibited excellent stability at pH 7.4 and underwent rapid destabilisation upon acidification [14, 15].

For polymeric delivery, few common examples of pH sensitive polymers are poly(methacrylic acid)s, poly(vinylpyridine)s, poly(vinylimmidazole)s.

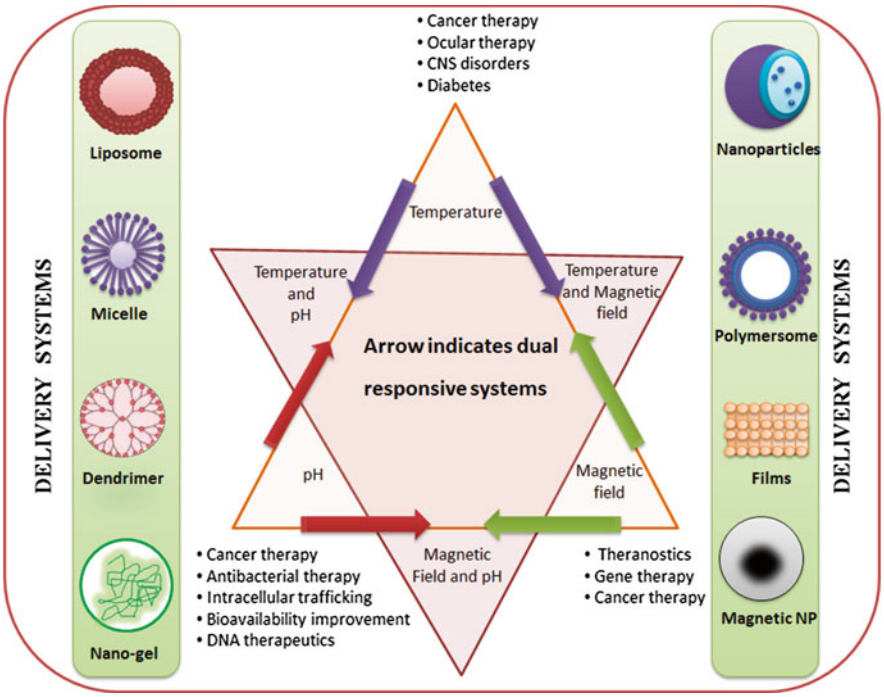

Fig. 10.2 Stimuli responsive carriers

Moreover, efforts are being made to develop new co-polymers with pH responsive properties. Giacomelli et al. Synthesised poly(ethyleneoxide)-b-poly(glycerolmonomethacrylate)–Indomethacin conjugates. This polymer–drug conjugate self assembled into micelle in water. The release of Indomethacin (IND) was governed by intrinsic molecular characteristics of free-IND (aqueous dissociation behavior) and pH-sensitivity of ester linkages in the conjugates. At neutral pH, the ester bond linkages were stable which promoted diffusion of free-IND out of the carrier, whereas acidic pH facilitates sustained release with slow kinetics [16].

pH sensitive poly(methacrylic acid and methacrylate) nanoparticles were designed to improve oral bioavailability of cyclosporin [17, 18]. Borchert et al. observed the pH-induced release of hydrophilic dyes from poly(2-vinylpyridine-b-ethylene oxide) block copolymer vesicles. At pH less than 5, protonation and dissolution of the poly-2-vinylpyridine blocks facilitated the release of dye from vesicles [19]. Similarly, gold-decorated shape-persistent, pH-responsive polymersomes were prepared by the self-assembly of a novel poly(ethyleneoxide)-block-poly[2-(diethylamino)ethylmethacrylate-stat-3-(trimethoxysilyl)-propylmethacrylate], copolymer. These pH-sensitive blocks are located in the membrane walls, while the hydrophilic PEO chains forms the corona [20].

Table 10.1 Polymers and systems used for different stimuli responsive carriers

S No.	Polymer	System	Outcome	Ref
pH responsive carriers				
1	Poly(L-glutamic acid)-b-poly(butadiene)	Polymersome	pH-triggered size change was observed	[106, 107]
2	Poly(ethylene glycol)-b-poly(glycerol monomethacrylate)-Indomethacin	Copolymer–drug conjugate	Acidic pH facilitated sustained release with slow kinetics	[16]
3	Poly(ethylene glycol)-b-poly(2-vinylpyridine)	Polymersome	Fluorescein/pH-triggered	[19]
4	poly(L-lysine)-b-poly(leucine)	Polymersome	Biodegradable pH responsive polymer	[108]
5	Poly(2-(methacryloyloxy) ethyl phosphorylcholine)-b-poly(2-(diisopropylamino) ethyl methacrylate)	Polymersome	DNA release at pH less than 6	[109]
6	Poly(ethylene glycol)-b-poly(styrene)-b-poly (2-diethylaminoethyl methacrylate)	Polymersome	pH-tunable membrane permeability	[100]
7	Poly(L-lysine)-b-poly(L-GLUTAMIC acid)	Micelles and vesicles	Biodegradable pH responsive polymer	[110]
8	Linear copolymers of ethylene and acrylic acid	Nanoparticles	Nanoparticles dispersed in aqueous media exhibited remarkable reversible thermoresponsive behaviour upon heating/cooling from 25 to 80 °C	[45]
9	Egg phosphatidylcholine and cholesteryl hemisuccinate	Liposome	These liposomes exhibited excellent stability at pH 7.4 and underwent rapid destabilization upon acidification	[14]
Thermal responsive carriers				
10	Poly(2-cinnamoylethyl methacrylate)-*block*-poly (*N-isopropylacrylamide*) (PCEMA-*b*-PNIPAM)	Vesicles	Thermosensitive release of drug 4-aminopyridine from the vesicles	[111]
11	Poly(ethylene glycol)-b-poly(N-isopropylacrylamide)	Vesicles	Doxorubicin-containing PEG–PNIPAM based vesicles, temperature induced transition allowed vesicle to release the drug	[56]
12	Poly(lactide)-b-poly(N-isopropylacrylamide)	Vesicles	Thermosensitive shell-cross-linked vesicles	[112]

13	Conjugated linoleic acid-coupled Pluronic F-127	Gel	For treatment of peritoneal metastasis of gastric cancer, Docetaxel was loaded into the gel	[57]
14	Poly(lactic acid-*co*-glycolic acid)-poly(ethylene glycol)-poly(lactic acid-c*o*-glycolic acid)	Injectable sol–gel formulation	Long-acting injectable formulation in treatment of type II diabetes, degradation of the polypeptide drug exenatide was reduced	[62]
15	Carboxymethyl hexanoyl Chitosan with glycerophosphate di-sodium salt and glycerol	Injectable nanogel	Loaded with hydrophilic anti-epilepsy drug ethosuximide	[64]
16	Chitosan β-glycerophosphate (β-GP) gel	Gel	Thermo-gelation was observed above 33 °C, for the treatment of Critical limb ischaemia	[113]
17	Polybenzofulvene derivative (poly-6-MOEG-9-BF3k)	Nano-aggregates	Sustained release of leuprolide was observed at 37 °C while at lower temperature burst release pattern was observed	[114]
Magnetically responsive carriers				
18	Polybutadiene-b*lock*-poly(glutamic acid)	Nano-composites	Magnetic nanocomposites of polybutadiene-b*lock*-poly(glutamic acid) combined with hydrophobically modified γ-Fe_2O_3 nanoparticles.	[78]
			Structurally found as filled micelles or hollow vesicles with a magnetic membrane	
19	Poly(ethylene glycol)-b-poly(2-vinylpyridine)/ Poly(ethylene glycol)-b-poly(isoprene)	Nanoparticles	Magnetic nanoparticles have been assembled into the bilayer membrane of block copolymer to form oligo-lamellar vesicles	[115]

10.3.2 Applications

10.3.2.1 Anti Cancer Therapy

pH sensitive liposomes made of dioleoylphosphatidylethanolamine (DOPE) and oleic acid or DOPE and 1,2-dipalmitoylsuccinylglycerol explored multiple possibilities to treat cancer. Problems associated with less circulation half-life, stability in blood have been resolved [21]. Recently, liposomal delivery system modified with pH-responsive cell penetrating peptide TH (TH-Lip) has been reported. TH was found to be a wonderful pH responsive ligand as the cell penetrating capacity of TH concealed during the blood circulation and in normal tissues at neutral pH. However, when TH-Lip reached the tumour, low pH at these sites promoted protonation of TH and the surface charge of TH-Lip converted from negative to positive thus promoted enhanced cellular and tumour spheroid uptake [22].

10.3.2.2 Antibacterial Therapy

Bacterial infections are generally characterised by very low pH values because of anaerobic fermentation and subsequent inflammation. In this regard, systemic antibiotic therapy was achieved by incorporating an ionisable polyhistidine segment in a block copolymer to make PLGA-b-polyhistidine-b-PEG triblock copolymer nanoparticles. A charge switch at the sites of localised acidity promoted interactions with the negatively charged bacterial wall, and led to increased nanoparticle uptake in both Gram-positive and Gram-negative bacteria [23].

10.3.2.3 Intracellular Trafficking

The usefulness of pH-sensitive liposomes has been well exhibited in a wide variety of applications, these include the transport of fluorescent probes to estimate the efficacy of different liposome compositions and to explain the mechanisms involved in intracellular trafficking, the intracellular transport of antigens, targeting intracellular pathways involved in processing and presentation of antigens and enhancing the immune response to tumour cells [24].

10.3.2.4 Oral Bioavailability Improvement

Because of the broad range of pH found throughout the gastrointestinal tract, pH-responsive systems for oral drug delivery have been designed to protect drugs from the harsh conditions found in the gastric cavity and to improve their absorption in the intestine [25]. For instance, poly(methacrylic acid)-based copolymers have been used as pH-sensitive coatings at the surface of porous silica nanoparticles, as well as to prepare copolymer micelles able to disassemble at the intestinal pH [26].

This charge-reversal approach was also applied to multi stimuli responsive nanocarriers to achieve drug release at neutral pH by taking advantage of electrostatic interactions, and to chitosan nanoparticles for gastric or intestinal delivery [27]. Similarly it was reported, pH sensitive oly(methacrylic acid and methacrylate) nanoparticles improved the oral bioavailability of cyclosporin [18].

10.3.2.5 DNA Therapeutics

Gene therapy is most widely explored field in biomedical research and increasing interest in stimuli responsive carriers to deliver DNA therapeutics can open multiple opportunities in this area. pH senstive liposomes were developed to deliver plasmid DNA into mammalian cell lines [28].

10.4 Thermoresponsive Carriers

Thermoresponsive carriers are usually governed by a nonlinear sharp change in the properties of at least one component of the nanocarrier materials with temperature (Fig. 10.2 and Table 10.1). Such a sharp response triggers the release of the drug following a variation in the surrounding temperature. Ideally, thermosensitive nanocarriers should retain their load at body temperature (~37 °C), and rapidly deliver the drug within a locally heated tumour (~40–42 °C) to counteract rapid blood-passage time and washout from the tumour [2]. The use of temperature as a signal has been justified by the fact that the actual body temperature often deviates from the physiological value (37 °C) in the presence of pathogens or pyrogens. This deviation can be a useful stimulus to activate release of therapeutic agents from various temperature-responsive drug delivery systems for diseases accompanied by fever. Drug-delivery systems responsive to temperature utilize various polymer properties, including the thermally reversible transition of polymer molecules, swelling change of networks, glass transition and crystalline melting [29].

Thermoresponsive polymers utilize subtle changes in temperature to trigger macroscopic changes in material properties. Polymers that possess a lower critical solution temperature (LCST) typically undergo a sol-gel phase transition when heated above their LCST, whereas polymers that become soluble upon heating are said to possess an upper critical solution temperature (UCST) [6]. Both systems can be exploited for drug delivery purposes. LCST copolymers can simply be mixed with drug as a liquid suspension at room temperature and delivered via minimally invasive injection techniques directly to hard-to-access target tissues within the body. Heating to physiologic temperature drives a sol–gel phase transition, which entraps the infused drug within a solid depot and can provide sustained release of therapeutic concentrations of drug directly at the site of interest [30]. Drug-releasing polymer systems possessing a UCST may employ temperature-induced swelling or scaffold destabilisation to rapidly release drug at a target site [31]. Localised heating (tumour tissues) or the application of an externally applied

stimulus (ultrasound, infrared laser and so on) may be utilised to induce the local destabilisation of a UCST drug-releasing copolymer scaffold to produce targeted release [32, 33].

Typical LCST polymers are based on *N*-isopropylacrylamide (NIPAM), *N*,*N-diethylacrylamide* (DEA), methylvinylether and *N*-vinylcaprolactam (NVCL) as monomers. Some example of these categories are Poly(*N*-vinylcaprolactam) [34], Poly(*N*-isopropylacrylamide) [35], Poly(*N*,*N*-ethylmethylacrylamide) [36], Poly(*N-ethylacrylamide*) [37], Poly(*N*,*N*-diethylacrylamide) [38]. A typical UCST system is based on a combination of acrylamide and acrylic acid [6]. Thermoresponsiveness can also occur on a brief temperature decrease (also called cold shock or cryotherapy). In this case, a thermally reversible swelling or de-swelling of the nanocarrier leads to free diffusion of the encapsulated drugs as a consequence of increased porosity [39]. Thermosensitive amphiphilic polymers generally have temperature-responsive hydrophilic segments and a suitable hydrophobic segment. NIPAM and its random copolymers are the most intensively investigated temperature-sensitive hydrophilic segments [40]. Block copolymers of PEG as a hydrophilic block and NIPAM or poly(*N*-isopropylacrylamide)-co-*N*-(2-hydroxypropyl) methacrylamide-dilactate as a thermosensitive block are able to self-assemble in water into temperature-responsive nanocarriers above the LCST of the thermosensitive block [41]. The hydrophobic segments, poly (L-lactide), cholic acid, alkyl, and poly (γ-benzyl L-glutamate) have also been used in diblock polymers with the temperature-sensitive polyacrylamide derivatives being the hydrophilic segments.

10.4.1 Delivery Systems

Generally used thermoresponsive carriers are liposomes, or polymer micelles, nanoparticles and nanofibres [42–48]. For liposomes, thermoresponsiveness usually arises from a phase transition of the constituent lipids and the associated conformational variations in the lipid bilayer [49]. In vivo, heat is generally applied by using temperature-controlled water sacks, radiofrequency oscillators or miniature annular-phased array microwave applicators. Liposome-embedded hydrogels have been widely used for controlled drug release. Liu et al. embedded egg phosphatidylcholine liposome into a poly(*N*-isopropylacrylamide) (pNIPAM) hydrogel via chemical cross-linking. It was found that the confinement of the network and the hydrophobic interactions between the liposome and pNIPAM modulated the integrity of the liposome and the release profile of the encapsulated content, such as calcein [50].

Polymeric micelles have been explored for temperature induced release of actives for drug and gene delivery. The temperature-sensitive property is possessed by the outer shell of the polymeric micelles and the drug molecules are incorporated into the hydrophobic inner core [1]. Co-polymer of pNIPAM and poly(acrylic acid) with LCST 33 °C had shown potential to be developed as novel injectable drug delivery system due to rapid sol to gel conversion upon subcutaneous injection [51].

Chang et al. developed a block co-polymer from pNIPAM and poly(methyl methacrylate). They prepared prednisone acetate loaded uncross-linked micelles and cross-linked micelles with newly developed block co-polymer. LCST of uncross-linked and cross-linked micelles were 31.0 and 40.8 °C respectively. Uncross-linked micelles showed a rapid drug release near to 30 °C while cross-linked (SCL) micelles displayed negligible release up to 37 °C which increased rapidly above 40 °C [52]. Thermoresponsive, self-assembling polymersomes of poly(*N*-[3-aminopropyl] methacrylamide hydrochloride) and pNIPAM were also synthesised and used for similar applications [53].

Vitamin B-12 loaded nanofibres of pNIPAM/poly(ethylene oxide) (PEO) blend were also able to program drug release with the variation of temperature. Fibres containing higher ratios of pNIPAM displayed rapid release below LCST while the prolonged release was observed at 37 °C [54]. Stover and coworkers developed thermoresponsive, biodegradable linear-dendritic nanoparticles for targeted and sustained release of a pro-apoptotic drug ceramide (C6). These nanoparticles showed preferential uptake into human MDA-MB-231 breast adenocarcinoma cells at temperature above the LCST (37 °C) and sustained release of C6 up to 1 month in vitro [55].

10.4.2 Applications

10.4.2.1 Cancer Therapy

Thermoresponsive drug delivery is among the most investigated stimuli-responsive strategies, and has been widely explored in oncology. Qin et al. prepared thermoresponsive, doxorubicin-containing PEG–pNIPAM based polymersomes. Temperature induced transition facilitated self-assembly of polymer into vesicles at temperatures above 32 °C. Temperature-controlled release was determined by incorporating a hydrophobic fluorescent dye into their membrane. These vesicles destabilised, or ruptured upon local cooling with either ice or penetrating cryoprobes [56]. For the treatment of gastric cancer, linoleic acid-coupled Pluronic F-127 (Plu-CLA) based thermoresponsive hydrogel loaded with docetaxel were developed. Docetaxel–Plu-CLA showed excellent anti-tumour activity, induced apoptosis and significantly reduced the number of peritoneal metastatic nodules than docetaxel alone [57].

10.4.2.2 Anti-Adhesive

Another application of thermosensitive polymers to inhibit ischemia-induced postoperative peritoneal adhesion was highlighted by Wu and coworkers. The PCL–PEG–PCL, developed for this purpose exhibited rapid micelle formation at 10 °C and sol to gel conversion at body temperature. They were found to be well tolerated, less toxic and therapeutically more effective as compared to control

group. Emergence of a layer of neo-mesothelial cells on the injured tissues after micelle treatment provides a strong evidence in the support of its anti-adhesion activity [58].

10.4.2.3 Temperature-Responsive Surfaces

Temperature-responsive cell culture surfaces of pNIPAM with the ability to alter its surface hydrophobicity in response to temperature were developed. The so developed cell culture surfaces facilitated cell adhesion and proliferation at 37 °C while released spread cultured cells below 32 °C without any need of trypsin. Further, pre-coating of these surfaces with fibronectin improved spreading of less adhesive cultured hepatocytes [59]. Liao et al. developed NIPAM-based thermoresponsive polyelectrolyte multilayer films as culture substrates to support hMSC expansion. These film were via layer-by-layer adsorption of thermoresponsive polymer and positively charged allylamine hydrochloride, or negatively charged styrene sulfonic acid. Surface charge was found to alter ECM structure and subsequently cellular response for the surface. The positively charged surfaces resulted improved cell adhesion and growth compared to control surfaces [60].

10.4.2.4 Diabetes Mellitus

For treatment of diabetes mellitus, pancreatic islet cells were harvested on laminin-5 coated temperature-responsive dishes functional activity of the islet cell sheets was confirmed by histological examination and Insulin secretion assay prior to in vivo transplantation [61]. Thermoreversible hydrogel composed of poly(lactic acid-co-glycolic acid)-poly(ethylene glycol)-poly(lactic acid-co-glycolic acid) (PLGA-PEG-PLGA) triblock copolymers loaded with exenatide were also evaluated to treat diabetes. Polymer decreased the degradation of the polypeptide. Further, the problems of loading of water soluble peptide and sustaining the release of peptide were solved by synergistic effect of zinc acetate, PEG, and sucrose [62].

10.4.2.5 Ocular Therapy

Thermosensitive poly(*N*-isopropylacrylamide)–chitosan (pNIPAM–CS) solution loaded with timolol maleate was investigated for ocular application due to its in situ gel-forming properties. Polymer showed lower critical solution temperature of 32 °C, which was close to the surface temperature of the eye. Ocular pharmacokinetic analysis on rabbit eye showed higher Cmax and AUC as compared to conventional eye drop solution. Moreover, pNIPAM–CS solution showed reduced in vitro cytotoxicity and higher capacity to reduce the intra-ocular pressure as compared to conventional solution [63]

10.4.2.6 CNS Disorders

Thermo-gelling injectable nanogels amphiphilically modified chitosan were reported for delivery ethosuximide. In vivo studies suggested prominent therapeutic effect of ethosuximide loaded nanogels by suppressing spike wave discharges in Long Evan rat model [64].

10.5 Magnetically Responsive Carriers

A magnetic field-responsive nanocarrier generally involves paramagnetic or superparamagnetic materials either embedded into a polymeric scaffold forming liposomal, micellar, or supramolecular aggregates (Fig.10.2 and Table 10.1). The versatile intrinsic properties of magnetic particles enable their use in numerous medical applications, such as: localisation of therapy, where magnetic carriers, associated with drugs, nucleic acids or loaded within cells can be directed or guided by means of a magnetic field gradient towards certain biological targets; magnetic fluid hyperthermia, where selective thermal ablation of tumours is achieved through heating of tumour-localised magnetic particles exposed to a high frequency magnetic field; tissue engineering, where particles can be used in remote actuation for control of cellular behaviour enabling development of functional tissue or to provide means for a patterned cell assembly and facilitated seeding of tissue engineered scaffold with functional cells; and MRI, where magnetic particles are used as contrast agents [65].

Magnetic systems for magnetic targeting that have been proposed or employed so far fall into two main classes. In one class, magnets external to the body provide both the field to magnetize the carrier and field gradients for targeting [66, 67]. However, the use of external magnets imposes serious limitations in targeting deep tissues as their field strength and field gradient decrease exponentially with the distance from the surface. The other class is based on a combination of external magnets and magnets (or magnetizable devices) implanted local to the target region. In the second class of systems, the external magnet would typically provide the magnetizing field for the carrier, while the local magnet (or magnetisable implant) will provide the largest possible field gradients for targeting. The second type of magnet system can be of potential use for targeting deep tissues, including blood vessels where magnetizable implants can be placed [68–70]. The effective use of magnetically responsive nanocarriers for biomedical applications such as targeted drug delivery depends on a number of factors related to the size and magnetism of the biocompatible nanoparticles. Parameters such as the physicochemical properties of the drug-loaded nanocarriers, field strength and geometry, depth of the target tissue, rate of blood flow, and vascular supply play a role in determining the effectiveness of this method of drug delivery [71, 72].

Iron oxides with core/shell structures are the most widely used as sources of magnetic materials. Iron oxides have several crystalline polymorphs known as α-Fe_2O_3 (hematite), β-Fe_2O_3, γ-Fe_2O_3 (maghemite), ε-Fe_2O_3, Fe_3O_4 (magnetite) and some others (amorphous and high pressure forms). Nevertheless, only maghemite and magnetite found the greatest interest of bio-applications [73]. Readily, carbonyl iron, which is well-known material with a unique form of elemental iron because of its small particle size, was also used as magnetic core [74]. In some reports, pure metals, such as Fe and Co were chosen as a magnetic material because they have several advantages over iron oxides, e.g. better magnetic properties, high saturation magnetisation, and high specific loss of power [75, 76].

Functionalisation of magnetically responsive carriers with amino group, silica, polymer, various surfactants or other organic compounds is usually provided in order to achieve better physical and chemical properties. Moreover, the core/shell structures of nanocarriers have the advantages of good dispersion, high stability against oxidation and appreciable amount of drug can be loaded to the polymer shell [77]. Lecommandoux et al. developed magnetic nanocomposites of polypeptide-based diblock copolymers of polybutadiene-block-poly(glutamic acid) in combination with hydrophobically modified γ-Fe_2O_3 nanoparticles [78]. Furthermore, lots of functional groups from polymers on the surface can be used for further functionalisation to get various properties [79]. It is favoured that magnetically responsive carriers retain sufficient hydrophilicity and, with coating, do not exceed 100 nm in size to avoid rapid clearance by reticuloendothelial system. It was found that surface functionalisation also plays the key role in nanoparticle toxicity [80].

10.5.1 Delivery Systems

Candidate nanosystems for such a therapeutic approach are core–shell nanoparticles (a magnetic core made of magnetite coated with silica or polymer) [81, 82], magneto-liposomes (maghemite nanocrystals encapsulated in liposomes) [83, 84] and porous metallic nanocapsules [85] (Fig. 10.2). A novel nanocarrier, containing functionalised magnetite (Fe_3O_4) core that was conjugated with drug via acid-labile hydrazone-bond and encapsulated by the thermosensitive chitosan-g-poly(*N-isopropylacrylamide-co-N*,*N*-dimethylacrylamide) was reported. Polymer exhibited a LCST of 38 °C below which the drug release response was appreciably low [86]. Antibody-conjugated magnetoliposomes for targeting cancer cells were also reported [87].

10.5.2 Applications

Magnetically responsive carriers are getting significant attention in the field of theranostics. Theranostics is the fusion of therapeutics and diagnostics to design individualised pharmacotherapy. Paramagnetic nanoparticles were initially used as contrast agents for magnetic resonance imaging (MRI) later on surface modification

of these nanoparticles introduced various functions for the nanoparticles to be used for both gene delivery and MR imaging. The combination based on the nanoparticles allows non-invasive monitoring of in vivo gene delivery with MRI and delivery of therapeutic genes [88, 89]. Such magnetofection experiments were generally performed using nanoassemblies with cationic coatings to condense nucleic acids, which resulted in higher transfection efficiencies under a permanent magnetic field. These technique led to improved effectiveness in the transfection of siRNA in vitro and/or in vivo when directed against prostate [90] and breast cancers [91], as well as in the gene transfer to oligodendrocyte precursors for neural repair [92].

10.6 Recent Advancements

10.6.1 Dual and Multi Stimuli Responsive Carriers

In an effort to further fine tune drug release and augment therapeutic efficacy of nanoparticulate drugs, sophisticated polymeric nanoparticles that respond to dual and multi-stimuli such as pH/temperature, pH/redox, pH/magnetic field, temperature/reduction, double pH, pH and diols, temperature/magnetic field, temperature/enzyme, temperature/pH/redox, temperature/pH/magnetic, pH/redox/magnetic, temperature/redox/guest molecules and temperature/pH/guest molecules have been aggressively pursued (Fig. 10.2) [93–96]. It should be noted that the responses take place either simultaneously at the same location or in a sequential manner in different settings and/or compartments. These dual and multi-stimuli responsive polymeric nanoparticles might on one hand offer unprecedented control over drug delivery and release leading to superior in vitro and/or in vivo anti-cancer potency, and on the other hand also facilitate nanoparticles preparation and loading of drugs under mild conditions [97]. These two and more stimuli are combined in order to: (1) facilitate preparation of nanoparticles under mild conditions through application of an external stimulus such as temperature and pH; (2) trigger drug release via application of an external stimulus such as magnetic field, ultrasonic, light and temperature; (3) trigger drug release or reverse deshielding of nanoparticles thereby enhancing tumour cell uptake of nanoparticulate drugs in a mildly acidic tumour microenvironment; and/or (4) boost intracellular drug release in tumour cells under endo/lysosomal pH and/or cytosolic reductive conditions.

Shim et al. developed a polymer containing sulfamethazine as the pH-responsive component, and poly(ε-caprolactone-co-lactide) (PCLA) in a triblock with PEG, PCLA–PEG–PCLA, as the thermosensitive moiety [98]. By controlling precise ratios between the two parts the co-polymer showed a reversible sol–gel–sol transition phase. At room temperature and pH 8 the polymer remained in a solution state, when the environment was altered to 37 °C and pH 7.4, i.e. normal physiological conditions, there was a rapid phase transition to a gel state. Once the gel was formed it remained stable and degraded over time without changing local pH levels [98, 99]. Despite the advantageous versatility of these systems, they often appear as too

Table 10.2 Clinical trials for thermal and magnetically responsive carriers

S.No.	Clinical trial	Indication	Carrier	Drug	NCT no.	Phase	Status
Thermal responsive carriers							
1	Phase 1/2 study of ThermoDox with approved hyperthermia in treatment of breast cancer recurrence at the chest wall	Breast cancer	Liposome	Doxorubicin	NCT00826085	I/II	Recruiting
2	A study of ThermoDox™ in combination with radiofrequency ablation (RFA) in primary and metastatic tumors of the liver	Hepatic and liver neoplasm	Liposome	Doxorubicin	NCT00441376	I	Completed
3	Phase 2 study of Thermodox as adjuvant therapy with thermal ablation (RFA) in treatment of metastatic colorectal cancer(mCRC) (ABLATE)	Colon cancer Liver metastasis	Liposome	Doxorubicin	NCT01464593	II	Terminated
4	Study of ThermoDox with standardized radiofrequency ablation (RFA) for treatment of hepatocellular carcinoma (HCC) (OPTIMA)	Hepatocellular carcinoma	Liposome	Doxorubicin	NCT02112656	III	Not started
5	MRI guided high intensity focused ultrasound (HIFU) and ThermoDox for palliation of painful bone metastases	Bone metastasis, breast and lung cancer	Liposome	Doxorubicin	NCT01640847	II	Not started
6	Phase 3 study of ThermoDox with radiofrequency ablation (RFA) in treatment of hepatocellular carcinoma (HCC)	Hepatocellular carcinoma	Liposome	Doxorubicin	NCT00617981	III	Ongoing
7	Temperature-sensitive liposomal doxorubicin and hyperthermia in treating women with locally recurrent breast cancer	Breast cancer	Liposome	Doxorubicin	NCT00346229	I	Terminated
8	Heat activated liposomal doxorubicin and radiofrequency ablation in treating patients with primary or metastatic liver tumors	Liver cancer, metastatic cancer	Liposome	Doxorubicin	NCT00093444	I	Not completed
Magnetically responsive carriers							
9	Ferumoxytol—Iron oxide nanoparticle magnetic resonance dynamic contrast enhanced MRI	Head and neck cancer	Iron oxide nanoparticle	Ferumoxytol	NCT01895829	0	Ongoing
10	Magnetic-targeted doxorubicin in treating patients with cancer metastatic to the liver	Cancer	Iron and carbon magnetic beads	Doxorubicin	NCT00041808	I/II	Completed

11	Safety and efficacy of doxorubicin adsorbed to magnetic beads	Hepatocellular carcinoma	Iron and carbon magnetic beads	Doxorubucin	NCT00054951	I/II	Unknown
12	Safety and efficacy of doxorubicin adsorbed to magnetic beads vs. iv doxorubicin in treating liver cancer	Hepatocellular carcinoma	Iron and carbon magnetic beads	Doxorubucin	NCT00034333	II/III	Terminated
13	Magnetic nanoparticle thermoablation-retention and maintenance in the prostate:a phase 0 study in men (MAGNABLATE I)	Prostate cancer	Magnetic nanoparticle	–	NCT02033447	0	Not started
14	High-field MRI iron-based contrast-enhanced characterization of multiple sclerosis and demyelinating diseases	Multiple sclerosis	Iron oxide nanoparticle	Feraheme Gadolinium	NCT01973517	–	Not started
15	Pre-operative nodal staging of thyroid cancer using ultra-small superparamagnetic iron oxidemagnetic resonance imaging (USPIO MRI): preliminary study	Metastatic medullary thyroid cancer	Iron oxide nanoparticle	Ferumoxytol	NCT01927887	–	Recruiting
16	Plasmonic photothermal and stem cell therapy of atherosclerosis versus stenting (NANOM PCI)	Coronary artery disease	Gold nano-particles with iron oxide-silica shells	–	NCT01436123	I	Completed
17	USPIO magnetic resonance imaging (MRI)	Cancer of lymph node	Iron oxide nanoparticle	Feraheme®	NCT01815333	–	Recruiting
18	Iron nanoparticle enhanced MRI in the assessment of myocardial infarction (IRNMAN)	Myocardial infarction (MI), Inflammation	Iron oxide nanoparticle	Ferumoxytol	NCT01995799	II	Recruiting
19	Pre-operative staging of pancreatic cancer using superparamagnetic iron oxide magnetic resonance imaging (SPIO MRI)	Pancreatic cancer	Iron oxide nanoparticle	Feraheme®	NCT00920023	IV	Ongoing
20	A validation study of mr lymphangiography using SPIO, a new lymphotropic superparamagnetic nanoparticle contrast	Bladder, Genito-urinary, prostate cancer	Iron oxide nanoparticle	Feraheme®	NCT00147238	–	Terminated
21	Ferumoxytol for magnetic resonance imaging of myocardial infarction	MI	Iron oxide nanoparticle	Ferumoxytol	NCT01323296	–	Unknown
22	Inflammatory cell trafficking after myocardial infarction	MI, inflammation	Iron oxide nanoparticle	–	NCT01127113	–	Unknown

complicated and many still remain as proofs of concept. To ascertain the viability of these strategies, evidence of the regulation of the response to each stimulus would be needed both in vitro and in vivo.

10.6.2 Breathing Vesicles

The breathing, in this context, can be defined as a highly reversible vesicle volume change by a factor of approximately 7, which was accompanied by diffusion of species into and out of the vesicles with a relaxation time of approximately 1 min. A three-layered vesicle system with pH-induced "breathing" feature was designed with triblock copolymer poly(ethylene oxide)-block-polystyrene-block-poly (2-diethylaminoethylmethacrylate). Self-assembly into vesicles was observed at a pH of 10.4. As the pH decreased, both the vesicle size and the thickness of all three layers increased. Progressive swelling of the middle layer with a decrease in pH below 6 induced cracking of the two outer layers and also a sharp increase of the vesicle size and the wall thickness. When pH reached up to 3.4, the vesicle size was found to be increased by a factor of 1.9 and the wall showed a cracked surface. These changes between pH 10.4 and 3.4 were highly reversible with the relaxation time of 1 min with marked repeatedly. The change in the wall structure dramatically helped to increase the wall permeability to water along with rate of proton diffusion from practically zero to extremely rapid [100].

Similarly, CO_2-responsive breathing vesicles were synthesised with block polymer poly(ethylene oxide)-poly(*N*-amidino)dodecyl acrylamide (PEO-b-PAD). PEO-b-PAD self-assembled in to micelle like structure in which the PEO portion formed hydrophilic exterior and the PAD portion formed hydrophobic interior. The amidine group in copolymer transformed into a charged amidinium species upon reaction with CO_2 which reverted back to its original form upon exposure to argon (Ar). It was confirmed by much larger intact vesicles with a diameter of 205.25 nm and strikingly increases volume by 83.5 % after CO_2 treatment for 20 min. These vesicles shrunk back to their initial size in the presence of Ar [101].

10.7 Challenges

Despite their responsive feature and versatility for drug delivery, thermoresponsive systems have some unanswered questions too. Co-polymeric systems provided ease to modulate the system properties but they are facing the problem of biocompatibility, biodegradability, reproducibility and tailored drug release as per requirement of site of action [51, 99]. Development of amphiphilic poly(asparagine) based polymers, Poly[α/β-(DL-aspartate isopropylamide)-co-(succinimide)] PCL-PEG copolymer had solved the biodegradability issue up to certain extent still, more in vivo studies are needed to ascertain their complete safety [58, 102–104].

While theranostics is the most recent advancement which utilizes the magnet stimuli as a component of diagnosis still the mismatch of dose required for imaging and therapy creates problem and require further optimisation. Further, the concentration required for imaging may create toxicity problems [105].

10.8 Clinical Status

Nanocarriers that are responsive to exogenous stimuli (temperature and magnetic field) have reached clinical stage as they are more promising. Endogenous triggers are indeed difficult to control because they may vary from one patient to another (such as the pH of a tumour or the presence of reducing agents in the blood circulation). Table 10.2 gives information regarding responsive carriers that are in different phases of clinical trials.

10.9 Conclusions

With greater understanding of physiological differences between normal and disease tissues and advances in material design, there is an opportunity to develop nanocarrier systems for target-specific drug delivery that will respond to local stimuli. They can shift the paradigm in the delivery of medicine and diagnostics. This chapter explained the role of pH, temperature and magnetic field responsive nanocarriers for targeted-drug delivery. However, compared to the amount of research being done in the field, relatively few medical nanotechnologies have made it to the market. Clear demonstrations of biocompatibility and including biodegradable components will make these materials even more attractive for in vivo applications. Furthermore, development and implementation of scalable, cost-effective fabrication techniques will help promote clinical translation. Together, intelligent materials and nanocarriers provide a versatile toolbox that we believe will revolutionize the future of modern medicine.

References

1. Ganta S, Devalapally H, Shahiwala A, Amiji M (2008) A review of stimuli-responsive nanocarriers for drug and gene delivery. J Control Release 126:187–204
2. Mura S, Nicolas J, Couvreur P (2013) Stimuli-responsive nanocarriers for drug delivery. Nat Mater 12:991–1003
3. Yatvin MB, Weinstein JN, Dennis WH, Blumenthal R (1978) Design of liposomes for enhanced local release of drugs by hyperthermia. Science 202:1290–1293
4. Fleige E, Quadir MA, Haag R (2012) Stimuli-responsive polymeric nanocarriers for the controlled transport of active compounds: concepts and applications. Adv Drug Deliv Rev 64:866–884

5. Mellman I, Fuchs R, Helenius A (1986) Acidification of the endocytic and exocytic pathways. Annu Rev Biochem 55:663–700
6. Schmaljohann D (2006) Thermo-and pH-responsive polymers in drug delivery. Adv Drug Deliv Rev 58:1655–1670
7. Yin Q, Shen J, Zhang Z, Yu H, Li Y (2013) Reversal of multidrug resistance by stimuli-responsive drug delivery systems for therapy of tumor. Adv Drug Deliv Rev 65:1699–1715
8. Tang R, Ji W, Panus D, Palumbo RN, Wang C (2011) Block copolymer micelles with acid-labile ortho ester side-chains: synthesis, characterization, and enhanced drug delivery to human glioma cells. J Control Release 151:18–27
9. Gao W, Chan JM, Farokhzad OC (2010) pH-responsive nanoparticles for drug delivery. Mol Pharm 7:1913–1920
10. Zhang L, Guo R, Yang M, Jiang X, Liu B (2007) Thermo and pH dual-responsive materials for controllable oil/water separation. Adv Mater 19:2988–2992
11. Pistolis G, Malliaris A, Tsiourvas D, Paleos CM (1999) Poly(propyleneimine) dendrimers as pH-sensitive controlled-release systems. Chem Eur J 5:1440–1444
12. Hui H, Xiao dong F, Zhong lin C (2005) Thermo-and pH-sensitive dendrimer derivatives with a shell of poly (N, N-dimethylaminoethyl methacrylate) and study of their controlled drug release behavior. Polymer 46:9514–9522
13. Yuan H, Luo K, Lai Y, Pu Y, He B, Wang G, Wu Y, Gu Z (2010) A novel poly (L-glutamic acid) dendrimer based drug delivery system with both pH-sensitive and targeting functions. Mol Pharm 7:953–962
14. Sudimack JJ, Guo W, Tjarks W, Lee RJ (2002) A novel pH-sensitive liposome formulation containing oleyl alcohol. Biochim Biophys Acta 1564:31–37
15. Torchilin VP, Zhou F, Huang L (1993) pH-sensitive liposomes. J Liposome Res 3:201–255
16. Giacomelli C, Schmidt V, Borsali R (2007) Nanocontainers formed by self-assembly of poly (ethylene oxide)-b-poly (glycerol monomethacrylate)-drug conjugates. Macromolecules 40:2148–2157
17. Dai J, Nagai T, Wang X, Zhang T, Meng M, Zhang Q (2004) pH-sensitive nanoparticles for improving the oral bioavailability of cyclosporine A. Int J Pharm 280:229–240
18. Wang XQ, Dai JD, Chen Z, Zhang T, Xia GM, Nagai T, Zhang Q (2004) Bioavailability and pharmacokinetics of cyclosporine A-loaded pH-sensitive nanoparticles for oral administration. J Control Release 97:421–429
19. Borchert U, Lipprandt U, Bilang M, Kimpfler A, Rank A, Peschka-Suss R, Schubert R, Lindner P, Forster S (2006) pH-induced release from P2VP-PEO block copolymer vesicles. Langmuir 22:5843–5847
20. Du J, Armes SP (2005) pH-responsive vesicles based on a hydrolytically self-cross-linkable copolymer. J Am Chem Soc 127:12800–12801
21. Hong MS, Lim SJ, Oh YK, Kim CK (2002) pH-sensitive, serum-stable and long-circulating liposomes as a new drug delivery system. J Pharm Pharmacol 54:51–58
22. Zhang Q, Tang J, Fu L, Ran R, Liu Y, Yuan M, He Q (2013) A pH-responsive helical cell penetrating peptide-mediated liposomal delivery system. Biomaterials 34:7980–7993
23. Radovic-Moreno AF, Lu TK, Puscasu VA, Yoon CJ, Langer R, Farokhzad OC (2012) Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics. ACS Nano 6:4279–4287
24. Karanth H, Murthy RSR (2007) pH-Sensitive liposomes-principle and application in cancer therapy. J Pharm Pharmacol 59:469–483
25. Wang X-Q, Zhang Q (2012) pH-sensitive polymeric nanoparticles to improve oral bioavailability of peptide/protein drugs and poorly water-soluble drugs. Eur J Pharm Biopharm 82:219–229
26. Qu W, Li Y, Hovgaard L, Li S, Dai W, Wang J, Zhang X, Zhang Q (2011) A silica-based pH-sensitive nanomatrix system improves the oral absorption and efficacy of incretin hormone glucagon-like peptide-1. Int J Nanomedicine 7:4983–4994
27. Yang YQ, Zheng LS, Guo XD, Qian Y, Zhang LJ (2010) pH-sensitive micelles self-assembled from amphiphilic copolymer brush for delivery of poorly water-soluble drugs. Biomacromolecules 12:116–122

28. Legendre J-Y, Szoka FC Jr (1992) Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. Pharm Res 9: 1235–1242
29. Bajpai AK, Shukla SK, Bhanu S, Kankane S (2008) Responsive polymers in controlled drug delivery. Prog Polym Sci 33:1088–1118
30. Li Z, Guan J (2011) Thermosensitive hydrogels for drug delivery. Expert Opin Drug Deliv 8:991–1007
31. Hoogenboom R, Lambermont-Thijs HML, Jochems MJHC, Hoeppener S, Guerlain C, Fustin C-A, Gohy J-F, Schubert US (2009) A schizophrenic gradient copolymer: switching and reversing poly (2-oxazoline) micelles based on UCST and subtle solvent changes. Soft Matter 5:3590–3592
32. Hirsch LR, Stafford RJ, Bankson JA, Sershen SR, Rivera B, Price RE, Hazle JD, Halas NJ, West JL (2003) Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance. Proc Natl Acad Sci 100:13549–13554
33. Rapoport N (2007) Physical stimuli-responsive polymeric micelles for anti-cancer drug delivery. Prog Polym Sci 32:962–990
34. Laukkanen A, Valtola L, Winnik FM, Tenhu H (2004) Formation of colloidally stable phase separated poly (N-vinylcaprolactam) in water: a study by dynamic light scattering, microcalorimetry, and pressure perturbation calorimetry. Macromolecules 37:2268–2274
35. Schild HG, Tirrell DA (1990) Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions. J Phys Chem 94:4352–4356
36. Cao Y, Zhu XX, Luo J, Liu H (2007) Effects of substitution groups on the RAFT polymerization of N-alkylacrylamides in the preparation of thermosensitive block copolymers. Macromolecules 40:6481–6488
37. Liu HY, Zhu XX (1999) Lower critical solution temperatures of N-substituted acrylamide copolymers in aqueous solutions. Polymer 40:6985–6990
38. Geever LM, Lyons JG, Higginbotham CL (2011) Photopolymerisation and characterisation of negative temperature sensitive hydrogels based on N, N-diethylacrylamide. J Mater Sci 46:509–517
39. Lee SH, Choi SH, Kim SH, Park TG (2008) Thermally sensitive cationic polymer nanocapsules for specific cytosolic delivery and efficient gene silencing of siRNA: swelling induced physical disruption of endosome by cold shock. J Control Release 125:25–32
40. Yoshida R, Uchida K, Kaneko Y, Sakai K, Kikuchi A, Sakurai Y, Okano T (1995) Comb-type grafted hydrogels with rapid deswelling response to temperature changes. Nature 374:240–242
41. Shenoy DB, Amiji MM (2005) Poly(ethylene oxide)-modified poly(ε-caprolactone) nanoparticles for targeted delivery of tamoxifen in breast cancer. Int J Pharm 293: 261–270
42. Abulateefeh SR, Spain SG, Thurecht KJ, Aylott JW, Chan WC, Garnett MC, Alexander C (2013) Enhanced uptake of nanoparticle drug carriers via a thermoresponsive shell enhances cytotoxicity in a cancer cell line. Biomater Sci 1:434–442
43. Cammas S, Suzuki K, Sone C, Sakurai Y, Kataoka K, Okano T (1997) Thermo-responsive polymer nanoparticles with a core-shell micelle structure as site-specific drug carriers. J Control Release 48:157–164
44. Chen M, Dong M, Havelund R, Regina VR, Meyer RL, Besenbacher F, Kingshott P (2010) Thermo-responsive core-sheath electrospun nanofibers from poly (n-isopropylacrylamide)/ polycaprolactone blends. Chem Mater 22:4214–4221
45. Kryuchkov VA, Daigle JC, Skupov KM, Claverie JP, Winnik FM (2010) Amphiphilic polyethylenes leading to surfactant-free thermoresponsive nanoparticles. J Am Chem Soc 132:15573–15579
46. McDaniel JR, Dewhirst MW, Chilkoti A (2013) Actively targeting solid tumours with thermoresponsive drug delivery systems that respond to mild hyperthermia. Int J Hyperth 29:501–510
47. Okuzaki H, Kobayashi K, Yan H (2009) Thermo-responsive nanofiber mats. Macromolecules 42:5916–5918

48. Wang C, Flynn NT, Langer R (2004) Controlled structure and properties of thermoresponsive nanoparticle-hydrogel composites. Adv Mater 16:1074–1079
49. Culver HR, Daily AM, Khademhosseini A, Peppas NA (2014) Intelligent recognitive systems in nanomedicine. Curr Opin Chem Eng 4:105–113
50. Liu Y, Li Z, Liang D (2012) Behaviors of liposomes in a thermo-responsive poly (N-isopropylacrylamide) hydrogel. Soft Matter 8:4517–4523
51. Hu Z, Xia X (2004) Hydrogel nanoparticle dispersions with inverse thermoreversible gelation. Adv Mater 16:305–309
52. Chang C, Wei H, Wu DQ, Yang B, Chen N, Cheng SX, Zhang XZ, Zhuo RX (2011) Thermoresponsive shell cross-linked PMMA-b-P(NIPAAm-co-NAS) micelles for drug delivery. Int J Pharm 420:333–340
53. Li Y, Lokitz BS, McCormick CL (2006) Thermally responsive vesicles and their structural "locking" through polyelectrolyte complex formation. Angew Chem Int Ed 45:5792–5795
54. Song F, Wang X-L, Wang Y-Z (2011) Poly (N-isopropylacrylamide)/poly (ethylene oxide) blend nanofibrous scaffolds: Thermo-responsive carrier for controlled drug release. Colloids Surf B: Biointerfaces 88:749–754
55. Stover TC, Kim YS, Lowe TL, Kester M (2008) Thermoresponsive and biodegradable linear-dendritic nanoparticles for targeted and sustained release of a pro-apoptotic drug. Biomaterials 29:359–369
56. Qin S, Geng Y, Discher DE, Yang S (2006) Temperature-controlled assembly and release from polymer vesicles of poly (ethylene oxide)-block-poly (N-isopropylacrylamide). Adv Mater 18:2905–2909
57. Bae WK, Park MS, Lee JH, Hwang JE, Shim HJ, Cho SH, Kim DE, Ko HM, Cho CS, Park IK (2013) Docetaxel-loaded thermoresponsive conjugated linoleic acid-incorporated poloxamer hydrogel for the suppression of peritoneal metastasis of gastric cancer. Biomaterials 34:1433–1441
58. Wu Q, Li L, Wang N, Gao X, Wang B, Liu X, Qian Z, Wei Y, Gong C (2014) Biodegradable and thermosensitive micelles inhibit ischemia-induced postoperative peritoneal adhesion. Int J Nanomedicine 9:727–734
59. Yamato M, Konno C, Kushida A, Hirose M, Utsumi M, Kikuchi A, Okano T (2000) Release of adsorbed fibronectin from temperature-responsive culture surfaces requires cellular activity. Biomaterials 21:981–986
60. Liao T, Moussallem MD, Kim J, Schlenoff JB, Ma T (2010) N-isopropylacrylamide-based thermoresponsive polyelectrolyte multilayer films for human mesenchymal stem cell expansion. Biotechnol Prog 26:1705–1713
61. Shimizu H, Ohashi K, Utoh R, Ise K, Gotoh M, Yamato M, Okano T (2009) Bioengineering of a functional sheet of islet cells for the treatment of diabetes mellitus. Biomaterials 30:5943–5949
62. Li K, Yu L, Liu X, Chen C, Chen Q, Ding J (2013) A long-acting formulation of a polypeptide drug exenatide in treatment of diabetes using an injectable block copolymer hydrogel. Biomaterials 34:2834–2842
63. Cao Y, Zhang C, Shen W, Cheng Z, Yu LL, Ping Q (2007) Poly(N-isopropylacrylamide)-chitosan as thermosensitive in situ gel-forming system for ocular drug delivery. J Control Release 120:186–194
64. Hsiao MH, Larsson M, Larsson A, Evenbratt H, Chen YY, Chen YY, Liu DM (2012) Design and characterization of a novel amphiphilic chitosan nanocapsule-based thermo-gelling biogel with sustained in vivo release of the hydrophilic anti-epilepsy drug ethosuximide. J Control Release 161:942–948
65. Sensenig R, Sapir Y, MacDonald C, Cohen S, Polyak B (2012) Magnetic nanoparticle-based approaches to locally target therapy and enhance tissue regeneration in vivo. Nanomedicine 7:1425–1442
66. Alexiou C, Schmid RJ, Jurgons R, Kremer M, Wanner G, Bergemann C, Huenges E, Nawroth T, Arnold W, Parak FG (2006) Targeting cancer cells: magnetic nanoparticles as drug carriers. Eur Biophys J 35:446–450

67. Lubbe AS, Alexiou C, Bergemann C (2001) Clinical applications of magnetic drug targeting. J Surg Res 95:200–206
68. Chorny M, Fishbein I, Yellen BB, Alferiev IS, Bakay M, Ganta S, Adamo R, Amiji M, Friedman G, Levy RJ (2010) Targeting stents with local delivery of paclitaxel-loaded magnetic nanoparticles using uniform fields. Proc Natl Acad Sci 107:8346–8351
69. Pislaru SV, Harbuzariu A, Agarwal G, Witt T, Gulati R, Sandhu NP, Mueske C, Kalra M, Simari RD, Sandhu GS (2006) Magnetic forces enable rapid endothelialization of synthetic vascular grafts. Circulation 114:I-314–I-318
70. Yellen BB, Forbes ZG, Halverson DS, Fridman G, Barbee KA, Chorny M, Levy R, Friedman G (2005) Targeted drug delivery to magnetic implants for therapeutic applications. J Magn Magn Mater 293:647–654
71. Chomoucka J, Drbohlavova J, Huska D, Adam V, Kizek R, Hubalek J (2010) Magnetic nanoparticles and targeted drug delivering. Pharmacol Res 62:144–149
72. Sun C, Lee JSH, Zhang M (2008) Magnetic nanoparticles in MR imaging and drug delivery. Adv Drug Deliv Rev 60:1252–1265
73. Tucek J, Zboril R, Petridis D (2006) Maghemite nanoparticles by view of Mössbauer spectroscopy. J Nanosci Nanotechnol 6:926–947
74. Reshmi G, Mohan Kumar P, Malathi M (2009) Preparation, characterization and dielectric studies on carbonyl iron/cellulose acetate hydrogen phthalate core/shell nanoparticles for drug delivery applications. Int J Pharm 365:131–135
75. Arias J, Loez-Viota M, Lopez-Viota J, Delgado AV (2009) Development of iron/ethylcellulose (core/shell) nanoparticles loaded with diclofenac sodium for arthritis treatment. Int J Pharm 382:270–276
76. Pouponneau P, Leroux J-C, Martel S (2009) Magnetic nanoparticles encapsulated into biodegradable microparticles steered with an upgraded magnetic resonance imaging system for tumor chemoembolization. Biomaterials 30:6327–6332
77. Hu FX, Neoh KG, Kang ET (2006) Synthesis and in vitro anti-cancer evaluation of tamoxifen-loaded magnetite/PLLA composite nanoparticles. Biomaterials 27:5725–5733
78. Lecommandoux S, Sandre O, Chécot F, Rodriguez-Hernandez J, Perzynski R (2005) Magnetic nanocomposite micelles and vesicles. Adv Mater 17:712–718
79. Parvin S, Matsui J, Sato E, Miyashita T (2007) Side-chain effect on Langmuir and Langmuir–Blodgett film properties of poly(N-alkylmethacrylamide)-coated magnetic nanoparticle. J Colloid Interface Sci 313:128–134
80. Shubayev VI, Pisanic Ii TR, Jin S (2009) Magnetic nanoparticles for theragnostics. Adv Drug Deliv Rev 61:467–477
81. Hua M-Y, Liu H-L, Yang H-W, Chen P-Y, Tsai R-Y, Huang C-Y, Tseng I, Lyu L-A, Ma C-C, Tang H-J (2011) The effectiveness of a magnetic nanoparticle-based delivery system for BCNU in the treatment of gliomas. Biomaterials 32:516–527
82. Zhang L, Wang T, Yang L, Liu C, Wang C, Liu H, Wang YA, Su Z (2012) General route to multifunctional uniform yolk/mesoporous silica shell nanocapsules: a platform for simultaneous cancer-targeted imaging and magnetically guided drug delivery. Chemistry 18: 12512–12521
83. De Cuyper M, Joniau M (1988) Magnetoliposomes. Eur Biophys J 15:311–319
84. Plassat V, Wilhelm C, Marsaud V, Manager C, Gazeau F, Renoir JM, Lesieur S (2011) Anti-estrogen-loaded superparamagnetic liposomes for intracellular magnetic targeting and treatment of breast cancer tumors. Adv Funct Mater 21:83–92
85. Zhang F, Braun GB, Pallaoro A, Zhang Y, Shi Y, Cui D, Moskovits M, Zhao D, Stucky GD (2012) Mesoporous multifunctional upconversion luminescent and magnetic "nanorattle" materials for targeted chemotherapy. Nano Lett 12:61–67
86. Yuan Q, Venkatasubramanian R, Hein S, Misra RDK (2008) A stimulus-responsive magnetic nanoparticle drug carrier: magnetite encapsulated by chitosan-grafted-copolymer. Acta Biomater 4:1024–1037
87. Shinkai M, Suzuki M, Iijima S, Kobayashi T (1995) Antibody-conjugated magnetoliposomes for targeting cancer cells and their application in hyperthermia. Biotechnol Appl Biochem 21:125–137

88. Ma X, Zhao Y, Liang XJ (2011) Theranostic nanoparticles engineered for clinic and pharmaceutics. Acc Chem Res 44:1114–1122
89. Wu X, Hu J, Zhou L, Mao Y, Yang B, Gao L, Xie R, Xu F, Zhang D, Liu J (2008) In vivo tracking of superparamagnetic iron oxide nanoparticle-labeled mesenchymal stem cell tropism to malignant gliomas using magnetic resonance imaging. J Neurosurg 108:320–329
90. Park JW, Bae KH, Kim C, Park TG (2010) Clustered magnetite nanocrystals cross-linked with PEI for efficient siRNA delivery. Biomacromolecules 12:457–465
91. Prijic S, Prosen L, Cemazar M, Scancar J, Romih R, Lavrencak J, Bregar VB, Coer A, Krzan M, Znidarsic A (2012) Surface modified magnetic nanoparticles for immuno-gene therapy of murine mammary adenocarcinoma. Biomaterials 33:4379–4391
92. Jenkins SI, Pickard MR, Granger N, Chari DM (2011) Magnetic nanoparticle-mediated gene transfer to oligodendrocyte precursor cell transplant populations is enhanced by magnetofection strategies. ACS Nano 5:6527–6538
93. Chiang WH, Ho VT, Huang WC, Huang YF, Chern CS, Chiu HC (2012) Dual stimuli-responsive polymeric hollow nanogels designed as carriers for intracellular triggered drug release. Langmuir 28:15056–15064
94. Jeong B, Gutowska A (2002) Lessons from nature: stimuli-responsive polymers and their biomedical applications. Trends Biotechnol 20:305–311
95. Morimoto N, Qiu X-P, Winnik FM, Akiyoshi K (2008) Dual stimuli-responsive nanogels by self-assembly of polysaccharides lightly grafted with thiol-terminated poly (N-isopropylacrylamide) chains. Macromolecules 41:5985–5987
96. Pan Y-J, Chen Y-Y, Wang D-R, Wei C, Guo J, Lu D-R, Chu C-C, Wang C-C (2012) Redox/pH dual stimuli-responsive biodegradable nanohydrogels with varying responses to dithiothreitol and glutathione for controlled drug release. Biomaterials 33:6570–6579
97. Cheng R, Meng F, Deng C, Klok H-A, Zhong Z (2013) Dual and multi-stimuli responsive polymeric nanoparticles for programmed site-specific drug delivery. Biomaterials 34: 3647–3657
98. Shim WS, Yoo JS, Bae YH, Lee DS (2005) Novel injectable pH and temperature sensitive block copolymer hydrogel. Biomacromolecules 6:2930–2934
99. Chan A, Orme RP, Fricker RA, Roach P (2005) Remote and local control of stimuli responsive materials for therapeutic applications. Adv Drug Deliv Rev 65:497–514
100. Yu S, Azzam T, Rouiller I, Eisenberg A (2009) "Breathing" vesicles. J Am Chem Soc 131:10557–10566
101. Yan Q, Zhou R, Fu C, Zhang H, Yin Y, Yuan J (2011) CO2-responsive polymeric vesicles that breathe. Angew Chem 123:5025–5029
102. Tanimoto F, Kitamura Y, Ono T, Yoshizawa H (2010) A versatile biodegradable polymer with a thermo-reversible/irreversible transition. ACS Appl Mater Interfaces 2:606–610
103. Watanabe E, Boutis GS, Sato H, Sekine S, Asakura T (2014) NMR studies of thermoresponsive behavior of an amphiphilic poly (asparagine) derivative in water. Polymer 55:278–286
104. Watanabe E, Tomoshige N, Uyama H (2007) New biodegradable and thermoresponsive polymers based on amphiphilic poly (asparagine) derivatives. Macromolecular symposia. Wiley Online Library. pp 509–514
105. McCarthy JR (2009) The future of theranostic nanoagents. Nanomedicine 4:693–695
106. Checot F, Lecommandoux S, Klok HA, Gnanou Y (2003) From supramolecular polymersomes to stimuli-responsive nano-capsules based on poly (diene-b-peptide) diblock copolymers. Eur Phys J E 10:25–35
107. Kukula H, Schlaad H, Antonietti M, Forster S (2002) The formation of polymer vesicles or "Peptosomes" by polybutadiene-block-poly (L-glutamate)s in dilute aqueous solution. J Am Chem Soc 124:1658–1663
108. Bellomo EG, Wyrsta MD, Pakstis L, Pochan DJ, Deming TJ (2004) Stimuli-responsive polypeptide vesicles by conformation-specific assembly. Nat Mater 3:244–248

109. Lomas H, Canton I, MacNeil S, Du J, Armes SP, Ryan AJ, Lewis AL, Battaglia G (2007) Biomimetic pH sensitive polymersomes for efficient DNA encapsulation and delivery. Adv Mater 19:4238–4243
110. Checot F, Rodriguez Hernandez J, Gnanou Y, Lecommandoux S (2006) Responsive micelles and vesicles based on polypeptide diblock copolymers. Polymer Adv Tech 17:782–785
111. Chen X, Ding X, Zheng Z, Peng Y (2006) Thermosensitive cross-linked polymer vesicles for controlled release system. New J Chem 30:577–582
112. Hales M, Barner Kowollik C, Davis TP, Stenzel MH (2004) Shell-cross-linked vesicles synthesized from block copolymers of poly (D, L-lactide) and poly (N-isopropyl acrylamide) as thermoresponsive nanocontainers. Langmuir 20:10809–10817
113. Hastings CL, Kelly HM, Murphy MJ, Barry FP, O Brien FJ, Duffy GP (2012) Development of a thermoresponsive chitosan gel combined with human mesenchymal stem cells and desferrioxamine as a multimodal pro-angiogenic therapeutic for the treatment of critical limb ischaemia. J Control Release 161:73–80
114. Licciardi M, Amato G, Cappelli A, Paolino M, Giuliani G, Belmonte B, Guarnotta C, Pitarresi G, Giammona G (2012) Evaluation of thermoresponsive properties and biocompatibility of polybenzofulvene aggregates for leuprolide delivery. Int J Pharm 438:279–286
115. Krack M, Hohenberg H, Kornowski A, Lindner P, Weller H, Forster S (2008) Nanoparticle-loaded magnetophoretic vesicles. J Am Chem Soc 130:7315–7320

Chapter 11
Prodrug Conjugate Strategies in Targeted Anticancer Drug Delivery Systems

Shashwat Banerjee, Kiran Todkar, Govind Chate, and Jayant Khandare

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADEPT	Antibody-directed enzyme prodrug therapy
AuNP	Gold nanoparticles
BCR	Breakpoint cluster region protein
BTK	Bruton's tyrosine kinase
CDK inhibitor	Cyclin-dependent kinase inhibitors
CLL	Chronic lymphocytic leukemia
CPT	Camptothecin
CTC	Circulating tumor cells
Dox	Doxorubicin
DTC	Disseminated tumor cells
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
ERK	Extracellular signal-regulated kinases
FISH	Fluorescence in-situ hybridization
FOL	Folic acid
FR	Folate Receptor
GDEPT	Gene-directed enzyme prodrug therapy
GnRH	Gonadotropin-releasing hormone
GRP78	Glucose-regulated protein 78
HCC	Hepatocellular carcinoma

S. Banerjee • K. Todkar
Actorius Innovations and Research (AIR), Pune, India

G. Chate • J. Khandare (✉)
Maharashtra Institute of Pharmacy, MIT Campus, Kothrud, Pune, India
e-mail: jayant.khandare@mippune.edu.in

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_11

HER2	human epidermal growth factor 2
HPLC	High-performance liquid chromatography
IGFR	Insulin-like growth factor receptor
KRAS	Kirsten rat sarcoma
LHRH	Luteinizing hormone releasing hormone
mAb	Monoclonal antibodies
MAPK	Mitogen-activated protein kinases
MCL	Mantle cell lymphoma
mCRC	Metastatic colorectal cancer
MDNS	Magneto-Dendritic Nano System
MDR	Multiple-drug resistance
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NSCLC	Non-small-cell lung cancer
PCR	Polymerase chain reaction
PDGFRβ	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placental growth factor
RCC	Renal cell carcinoma
RET	Rearranged during transfection
RGD	Arginine-glycine-aspartic acid
SCCHN	Squamous cell carcinoma of the head and neck
scFv	Single chain variable fragment
siRNA	Small interfering RNA
Tf	Transferrin
Tf-PEG-AD	Transferrin-conjugated poly (ethylene glycol)-adamantane
TfR	Transferrin receptor
TPGS	D-α-Tocopheryl polyethylene glycol succinate
US FDA	United States Food and Drug administration
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

11.1 Introduction

The primary dearth of treatment in chemotherapy is lack of molecular selectivity and severe toxicity associated from an anticancer drug. In general, chemotherapeutic drugs responds through anti-proliferative mechanisms; or by preventing cell cycle at a specific phases rather than producing a toxic effect to particular site or types of cancer cells [1]. Hence, for the effective anticancer therapy, polymeric prodrug conjugation methodology represents one of the most promising approaches in achieving selective chemotherapy. Many efforts in reducing the systemic toxicity of chemotherapeutic moieties have been clinically explored (Fig. 11.1). In particular, polymeric prodrug conjugate is a chemical modification of a biologically inert

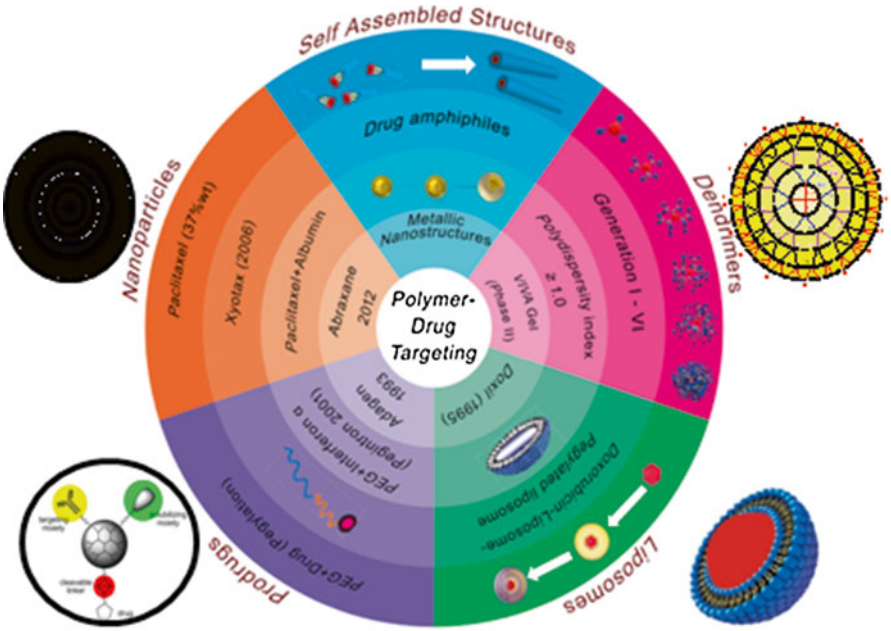

Fig. 11.1 Advances in drug delivery systems representing varied architectures, physicochemical traits such as size, shape, and surface charge (modified from [6])

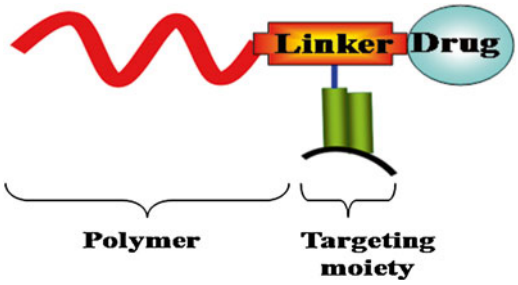

Fig. 11.2 Schematic representation of a polymeric prodrug conjugate with targeting moiety

component which is transformed to its active form, in vivo [2, 3]. Polymeric forms (e.g., poly(ethylene glycol) (PEG)) commonly referred in literature as "PEGylation" is a polymeric prodrug approach, offering an important tool to enhance the pharmacodynamics (PD) of the active pharmacologic component via a simple chemical alteration. Traditional prodrug design aims to offer: (1) enhanced aqueous solubility, chemical stability, brain permeability, and oral or local absorption of a drug; and (2) reduced undesired pre-systemic metabolism, and toxicity [4, 5].

An ideal polymeric prodrug conjugate system typically consists of multiple components as represented in Fig. 11.2.

1. A polymer as a drug delivery vehicle
2. Drug, protein or peptide as a biological active component
3. A spacer molecule and targeting moiety

Following approaches are generally used to target anticancer prodrugs to the tumor or cancer cells [7, 8]: (1) Passive Drug targeting and (2) Active Drug targeting.

Passive Drug Targeting: In passive targeting, drug is delivered to the targeted site by conjugating it with polymer which releases the drug outside the targeted site due to altered environmental conditions as represented in Fig. 11.3a. The general features of tumors and many inflamed areas of body include leaky blood vessels and poor lymphatic drainage which passively provides increased retention of macromolecules into tumor [9–12]. This phenomenon is commonly referred to as Enhanced Permeability and Retention (EPR) effect [9]. EPR effect is primarily a passive targeting due to the accumulation of prodrug, into the tumor. The phenomenon mainly occurs owing to hampered lymphatic drainage which allows them to release the drug into the tumor milieu. However, passive targeting approach has several limitations. This is because targeting of the cancer cells is not always achieved as the diffusion of some drugs is insufficient and the random chemical approach makes it difficult to control the process. The lack of control is expected to lead into multiple-drug resistance (MDR). This situation results in resistance of cancer cells towards one or more drugs, thereby leading to failure of chemotherapy treatments. Moreover, it is known that certain tumors do not show the EPR effect, and the permeability of vessels is unpredictable throughout tumor which further limits the passive targeting approach [13]. On the other hand, the more efficient way to obtain targeting is by "active targeting" process.

Active Drug Targeting: Active targeting approach involve interactions between specific biological systems, e.g., ligand–receptor, antigen–antibody, enzyme–substrate (Fig. 11.3a [14]). Active targeting is achieved by targeting ligand molecules that may interact with specific receptors on the cell surfaces—along with the bioactive prodrug system (Fig. 11.3b), designed by variety of synthetic conjugation methods. Most commonly used targeting components are small organic molecules, antibodies (mAbs), peptide ligands, sugar residues, aptamers (specific to particular receptors), selectins, antigens, and mRNAs overexpressed in targeted cells or organs. It is imperative that the targeting moiety binds with high selectivity to molecular receptors that are uniquely overexpressed on the cell surface.

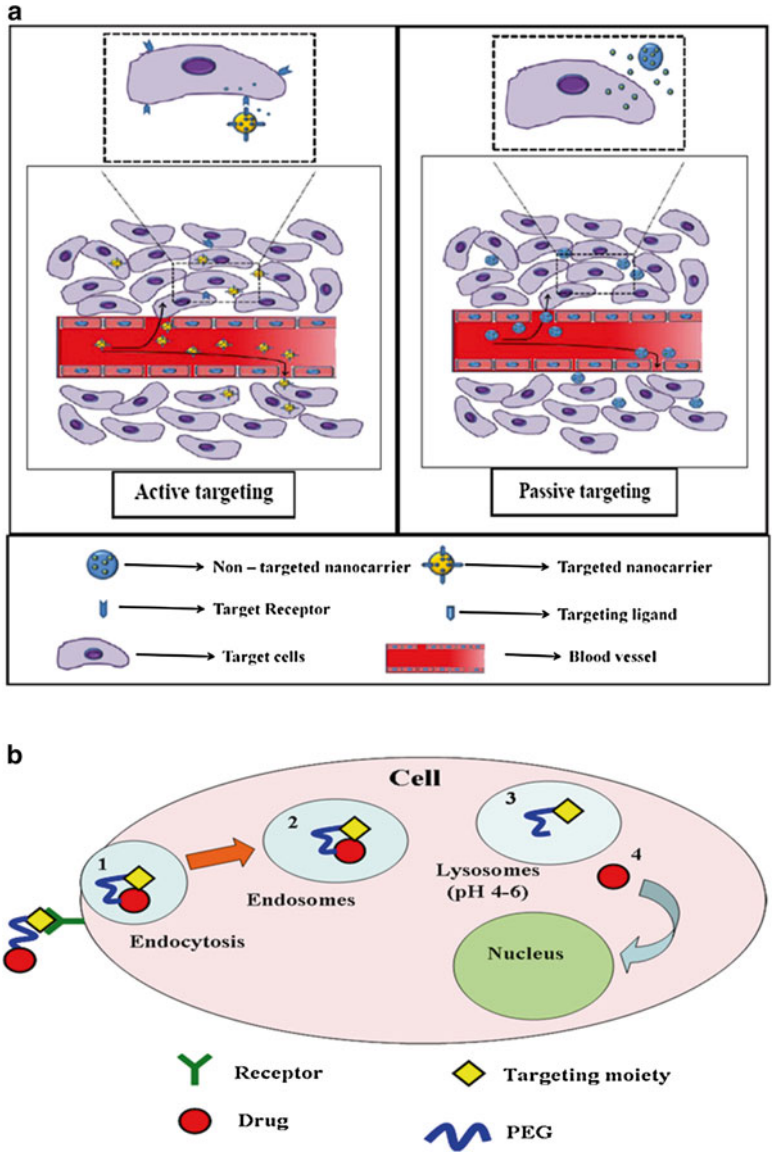

Fig. 11.3 (**a**) Active and passive targeting approaches though prodrug system, (**b**) (1) prodrug is docked at cell surface by ligand–receptor interaction and then internalized by tumor cells through receptor-mediated endocytosis, (2) transport of prodrug in membrane limited organelles, (3) fusion with lysosomes, (4) finally, drug is released intracellularly on exposure to lysosomal enzymes or lower pH (pH 6.5 to <4.0) [15, 16]

11.2 Prodrug Systems for Targeted Drug Delivery

The critical requirements for achieving selective targeting of prodrug to tumors are as follows: (1) it should be highly stable in blood circulation, (2) higher biodistribution to the targeted site, (3) adequate contact time with the target, (4) sufficient retention by the target, (5) retention of drug potency, and (6) adequate clearance fate of non-targeted compound [17]. Polymer therapeutics with various polymeric architectures have been reported to achieve cellular targetability and EPR effect (Fig. 11.4). Similarly, Table 11.1 shows different types of targeting through ligands, and their specific targets through various drug delivery systems. Furthermore, to target specific biological molecules (e.g., enzymes, peptide transporters, antigens) that are overexpressed in tumor cells in comparison to normal cells, new promising anticancer prodrugs can be designed which includes:

1. Enzyme-activated prodrugs—antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT) [17].
2. Targeting-ligand conjugated prodrugs—antibody–drug conjugates, peptide–drug conjugates, aptamer–drug conjugates, and folic acid–drug conjugates [18].
3. Enzyme-cleavable prodrugs.
4. Membrane transporter-associated prodrugs.
5. Polymeric prodrug conjugates.

The section below describes the classification and mechanisms of targeting.

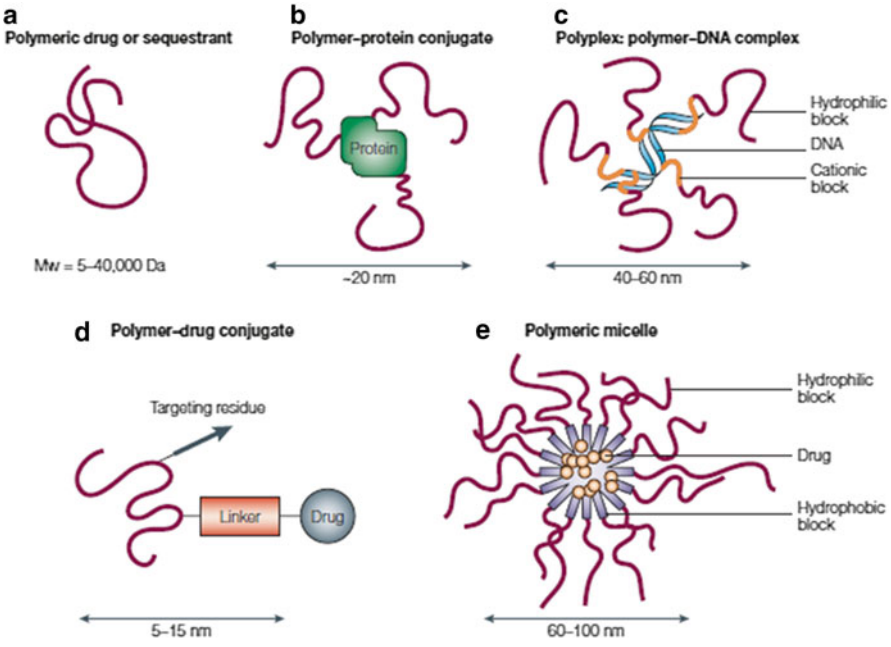

Fig. 11.4 (**a**-**e**) Different polymer therapeutics with various architectures for delivering biological actives (reproduced from [3])

Table 11.1 Examples of targeted molecular therapeutics approved by US FDA 1988–2014 (US FDA website [20], modified from [21])

Targeted molecular therapeutics	Brand name	Drug type	Type of cancer	Primary molecular targets	Year
Ibrutinib	Imbruvica	Small molecule	CLL, MCL	BTK, BCR	2014
Bevacizumab	Avastin	Monoclonal antibody	mCRC	VEGF Ligand	2013
Axitinib	Inlyte	Small molecule	Renal cell carcinoma	KITm PDGFRβ, VEGFR 1/2/3	2013
Sorafenib	Nexavar	Small molecule	HCC; RCC, DTC	RAF/MEK/ERK, VEGFR/ PDGFR	2013
Crizotinib	Xalkori	Small molecule	NSCLC	HGFR, c-MET, c-ros, RON	2013
Ziv-aflibercept	Zaltrap	Recombinant fusion protein	Colorectal cancer	PIGF, VEGFA/B	2012
Everolimus	Afinitor	Small molecule	RCC, breast cancer	mTOR	2012
Vandetanib	Caprelsa	Small molecule	Medullary thyroid Cancer	EGFR (HER1/ERBB1), RET, VEGFR2	2011
Sipuleucel-T	Provenge	Vaccine	Castrate-resistant prostate cancer	GnRH, LHRH	2010
Ofatumumab	Arzerra	Monoclonal antibody	CLL	ADCC, CDC	2009
Vorinostat	Zolinza	Small molecule	Cutaneous T-cell lymphoma	HDAC	2006
Cetuximab	Erbitux	Monoclonal antibody	SCCHN	EGFR, ADCC, KRAS	2004
Bortezomib	Velcade	Small molecule	Multiple myeloma, MCL	Proteasome	2003
Trastuzumab	Herceptin	Monoclonal antibody	Breast cancer, colon cancer	HER-2	1998
Methotrexate	Trexall	Small molecule	Breast, head and neck, leukemia	Folic acid	1988

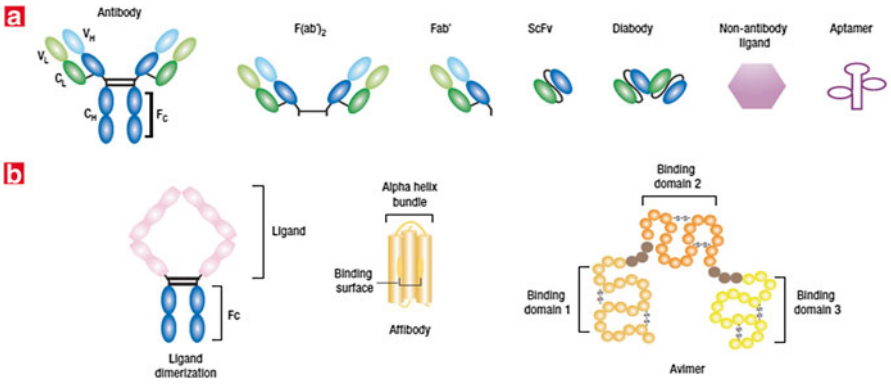

Fig. 11.5 (**a**) Various targeting molecules such as a monoclonal antibody or antibodies' fragments, non-antibody ligands, and aptamers. (**b**) Affinity and selectivity can be increased by dimerization of ligand or by screening for conformation-sensitive targeting agents such as intact antibodies and their fragments as well as antibodies, avimers and nanobodies (reproduced from [19])

11.3 Type of Targeting Moieties

Targeting agents can be classified broadly as proteins (mainly antibodies and their fragments), nucleic acids (aptamers), or other receptor ligands (peptides, vitamins, and carbohydrates) as shown in Fig. 11.5.

11.4 Implications of Molecular Targeting in Anticancer Therapy (e.g., CDK Inhibitors, mTOR, IGFR, VEGF)

The implementation of targeted cancer therapy for individual patient has revolutionized the existing ways for cancer therapy. There is an increasing importance of targeted therapy in the treatment of several cancer entities (e.g., colon, NSCLC, breast, lymphoma, and malignant melanoma) and its molecular targets such as human epidermal growth factor 2 (HER2) [22], epidermal growth factor receptor (EGFR) [23], cyclin-dependent kinase inhibitors (CDK inhibitor) [24], vascular endothelial growth factor (VEGF), etc. [25, 26]. However, the key issue in implementing targeted therapy may only be effective when the tumor carry certain molecular features; otherwise they can be ineffective or can create unwanted side effects. For example, panitumumab is active against colon cancer only when the tumor is Kirsten rat sarcoma viral oncogene (KRAS) wild type [27].

In order to avoid such limitations and designing diagnostic analysis of solid and hematological tumors, identifying target genes is an essential step. In order to have an identification process, the laboratories explore high-end techniques, such as

FISH assay, PCR, HPLC, protein arrays, DNA/RNA-array technology which are used to precisely detect the genetic alterations in the diseased state. For example, breast cancer is characterized by overexpression of HER2 which has been known to be more aggressive disease progression and a poorer prognosis [28, 29]. Hence, many researchers have focused on HER2 inhibitors as potential anticancer target which is achieved through gene therapy or by using drugs as Trastuzumab [30]. However, patients with HER2 positive breast cancer developed resistance towards the first FDA-approved therapeutic antibody for metastatic breast cancer "Trastuzumab" through hyperactivation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway [31]. The PI3K/Akt/mTOR signaling cascade has important regulatory functions in normal and oncogenic cellular growth, survival, proliferation, migrations and metabolism [32]. Several studies have shown that second mutations in this signaling pathway confers resistance mechanism to HER2-targeted therapies and direct inhibition of PI3K/Akt/mTOR signaling cascade may overcome trastuzumab resistance [33, 34]. Hence, combination of Trastuzumab and Anastrozole is targeted towards MAPK pathways and Akt pathway [35]. Likewise, inhibition of mTOR with drug Everolimus is efficacious when combined with Trastuzumab [36]. Recently, US FDA has approved Trastuzumab with Emtansine as a first antibody-drug conjugate for treating HER2-positive metastatic breast cancer [20]. In addition to many other molecular targets, sialic acid, a derivative of neuraminic acid, is one of the major targets in developing therapeutic treatment in cancer patients as hypersialylation has been shown to contribute cancer cell progression and metastasis [37]. However, till date, there is no therapeutic drug developed to interfere with sialic acid synthesis which might offer better treatment approach for cancer patients. Sialic acid as a targeting moiety with PEGylated doxorubicin (Dox) targeted prodrug conjugate demonstrated significant antitumor activity compared to free Dox and non-targeted conjugate counterpart. This significant effect is achieved by enhancing the permeation and prodrug uptake by cancer cells and cytotoxicity of the prodrug [38].

11.5 Role of Antibodies in Targeted Therapy

In 1975, Köhler and Milstein developed methods to recover antibodies in large amount which can be directed against specific antigens. Since then monoclonal have emerged as most important ligands for delivering contrast agents and chemotherapeutics for several different malignancies [39]. Monoclonal antibody are stable in blood and typically have nanomolar affinities for their target. The binding and non-binding domains of mAb are separated physically; hence, they could impart substitution with other chemical agents like, contrast agents and chemotherapeutics. Three targeting moieties, whole mAb, Fab′, and single chain variable fragment (scFv) were evaluated for targeting the same B-cell antigen CD19 [18]. The Fab′ immunoliposomes even though exhibited the most prolonged circulation times, exhibited statistically insignificant numbers of long-term survivors [18]. While, in

B-cell model, the anti-CD19 Fab′ immunoliposomes demonstrated increased circulation time and higher survival rates for Namalwa-bearing SCID mice as compared to the anti-CD19 mAb immunoliposome treatment [40].

More recently, the fragments of antibody containing only the variable region of the antibody are used for active targeting of therapeutics because they retain the specificity for their target [36]. In addition, they prevent complement activation due to the lack of constant Fc effector region or undesirable interaction with other cells. Furthermore, the smaller sizes of antibody fragments are important factor in the development of an actively targeting nanoparticle. Moreover, using antibody fragments can also help in efficient cell permeability [41]. Therapeutic agents must cross various biological barriers as well as the high interstitial pressure to reach their target cells. Towards this antibody fragments such as scFv and Fab are known to represent higher efficiency in penetrating tumor cells compared to intact antibody.

Immunogenicity caused by these antibodies is yet another important factor in using them for therapeutic targeting. Animal originated antibodies are obviously identified as foreign agents resulting in strong immune responses. However, genetic engineering tools can now design chimeric mouse-human mAbs. For example the anti-CD20 mAb rituximab (Rituxan) has revolutionized lymphoma treatment. On the other hand, humanized antibodies, containing only the binding regions of the mouse antibodies combined with a human antibody, exhibit reduction in immunogenicity [42]. However, they have shown reduced affinity in some cases. Further, antibodies could lose activity when translated into a conjugated form. Therefore, novel chemical strategies are essential to retain their potency even after the conjugation and at the same time are able to release the cytotoxic agent, either after binding to the cancer cell surface or after endocytosis into the cell. An example of such a conjugation is that of calicheamicin, a cytotoxic drug, conjugated to a tumor-targeting mAb through an amide linkage. The conjugate accumulates in the tumor but shows no appreciable cytotoxicity [43]. However, when calicheamicin is conjugated using a pH-sensitive bifunctional linker that permits its release intracellularly (Fig. 11.6), the conjugate shows potent antitumor activity [44].

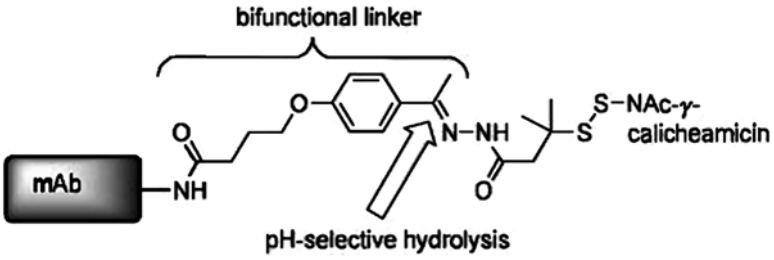

Fig. 11.6 Cleavable bifunctional linker for the conjugation of calicheamicin to monoclonal antibodies (reproduced from [17])

The prodrug was designed by coupling calicheamicin to various hydrazide bearing spacers through a disulfide bond, and the resulting moiety was conjugated to humanized anti-CD33 monoclonal antibody (clone P67.6) hinge region. Monoclonal antibody conjugated calicheamicin was then coupled to aldehyde-bearing oxidized carbohydrates, through hydrazone bonds [44]. In vivo study showed that active prodrugs rapidly cleaved at pH 4.5 whereas, they were stable at pH 7.4. On the other hand the efficacy of the prodrug system was found to be far less when calicheamicin was conjugated with a linker encompassing a non-pH-labile amide bond to mAb. This conjugate although had the same affinity for CD33 as Mylotarg, the system was found to be hundreds of times less cytotoxic in vitro and considerably less active in vivo and ex vivo [44]. In this system, the release of calicheamicin was believed to be due to intracellular oxidation of a hindered disulfide bond. This clearly indicated that the importances of linker chemistry for effective intracellular activation of calicheamicin–mAb conjugate.

11.6 Protein and Peptide Based Carrier Systems

Peptide ligands are being explored against a tumor-specific antigen or a peptide transporter that is overexpressed in cancer cells [40]. Peptide ligands can be directly conjugated to chemotherapy drugs to achieve a targeted delivery to cancer cells. Compared to antibody, peptides are more suitable targeting moieties because of (1) low molecular weight, (2) exceptional cell permeability, (3) ease in chemical conjugation, and (4) simple to produce [45].

The main approach in identifying appropriate peptide ligands is to screen peptide libraries produced by either phage display [46] or by chemical synthesis process [47, 48]. Phage display assists in identifying peptides that target a specific receptor, or certain cell types even if the receptors are unidentified [49]. Till date, various types of receptors or cells, have been discovered such as integrin receptors [50, 51], thrombin receptors [52], tumor cells [53–55], cardiomyocytes [56], and pancreatic β cells [57]. Tumor-targeting peptides have been effectively used in delivery vehicles for targeting small molecule drugs, oligonucleotides, liposomes, imaging agents, and inorganic nanoparticles to tumors. Furthermore, peptidomimetic self-assembled nanoparticles and peptide aptamers, which are peptide-related nanoparticles, also have shown great promise in targeted drug delivery. The former have wider applications in tumor imaging, tumor targeting delivery and vaccination [58], whereas the latter are directly used as drugs interfering with the function of receptors [59]. The use of these peptides has assisted in enhancing the specificity and efficacy of drug delivery with reduced side effects [60, 61]. The current discoveries of tumor lymphatic vessel targeting peptides present another route for targeted drug delivery for tumors [62–64].

Transferrin (Tf), a serum non-heme iron binding glycoprotein is a very pertinent targeting agent for cancer therapeutics due to overexpressed Tf receptors (TfR^+) on malignant cells compared to normal cells because of the higher demand for faster

cell growth and division. We and many others have demonstrated use of Tf for targeting TfR^+ in cancer therapeutics and diagnostics [65]. Tf is also extensively reported in human clinical trials with adriamycin [66], cisplatin [67], and diphtheria toxin [68].

An important study to demonstrate Tf mediated targeting was explored using 0.05 % PEG-AD containing Tf formulation [69]. The formulation was compared against AD-PEG-particles (with 0.0 % Tf-PEG-AD) using cells K562 for gene delivery. Tf-PEG-AD demonstrated a fourfold increase in transfection mainly due to Tf mediated uptake. However, presence of excess Tf added to 0.05 % Tf-PEG-AD particles eliminated the transfection enhancement [69].

An important characteristic of Tf ligand for targeting TfR^+ is the iron-binding efficiency. Transferrin ligand with low iron-binding efficiency resulted in a lower efficiency in binding to the TfR [68]. This was revealed from the flow cytometry study involving fluorescein-labeled transferrin (Tf-fluor) and holo-transferrin (holo-Tf, native transferrin), Tf-PEG-AD, or Tf-$(PEGAD)_2$. The Tf-PEG-AD and Tf-$(PEG\text{-}AD)_2$ showed lower binding affinities, while holo-Tf treatment showed the highest binding affinity mainly due to their oxidized state. As a result, a different synthesis route for Tf-PEG-AD was designed to improve the binding affinity to the Tf receptor. The synthesized Tf-PEG-AD nanoparticles (250 nM Tf-fluor/75 nM Tf-PEG-AD nanoparticles) showed a 15 % reduction in fluorescence compared to Tf-PEG-AD conjugates (250 nM Tf-fluor/75 nM Tf-PEG-AD in conjugate form) revealing high binding. The difference in receptor binding was mainly due to multiple interactions between each ligand-modified particle and cell surface receptors.

Davis et al. have also performed a novel study of small interfering RNA (siRNA) delivery in nonhuman primates using Tf-conjugated liposomes [70]. The efficacy of these Tf-conjugated liposomes had been proven effective in metastatic mouse models of Ewing's sarcoma, and consequently, the safety of the administration of these particles in nonhuman primates was the focus of this study [71, 72].

Interestingly, Tf-conjugated liposomes co-encapsulating Dox and verapamil (Tf-L-DOX/VER) have been shown to effectively overcome multi-drug resistance [73]. Cellular uptake of Tf-L-DOX/VER was 5.2 and 2.8 times greater with cytotoxicity (IC_{50} = 4.18 μM) than non-targeted liposomes having Dox and verapamil (IC_{50} = 21.7 μM) and Tf-conjugated liposomes loaded with Dox alone (IC_{50} = 11.5 μM) in a chronicmyelogenous leukemia cell line (K562 cells). In addition, the difference in cytotoxicity between the targeted and non-targeted liposomes was diminished within the presence of 2 mg/mL free Tf. This exhibits the effectiveness of the Tf moiety for cellular uptake and cytotoxicity [72].

We have recently shown that Tf conjugated multicomponent magneto-dendritic nanosystem (MDNS) can be efficiently used for rapid tumor cell targeting, isolation, and high-resolution imaging by a facile bioconjugation approach [65]. The bio-functionalized MDNS designed by combining multiple components such as Tf, iron oxide (Fe_3O_4) nanoparticles, fourth generation (G4) dendrimers, cyanine 5 NHS (Cy5) fluorescent NIR dye and glutathione (GSH) was able to capture TfR overexpressed cancer cells from an artificial circulating tumor cell (CTC)-like suspension (Fig. 11.7). The MDNS platform exhibited rapid capture (~5 min) of

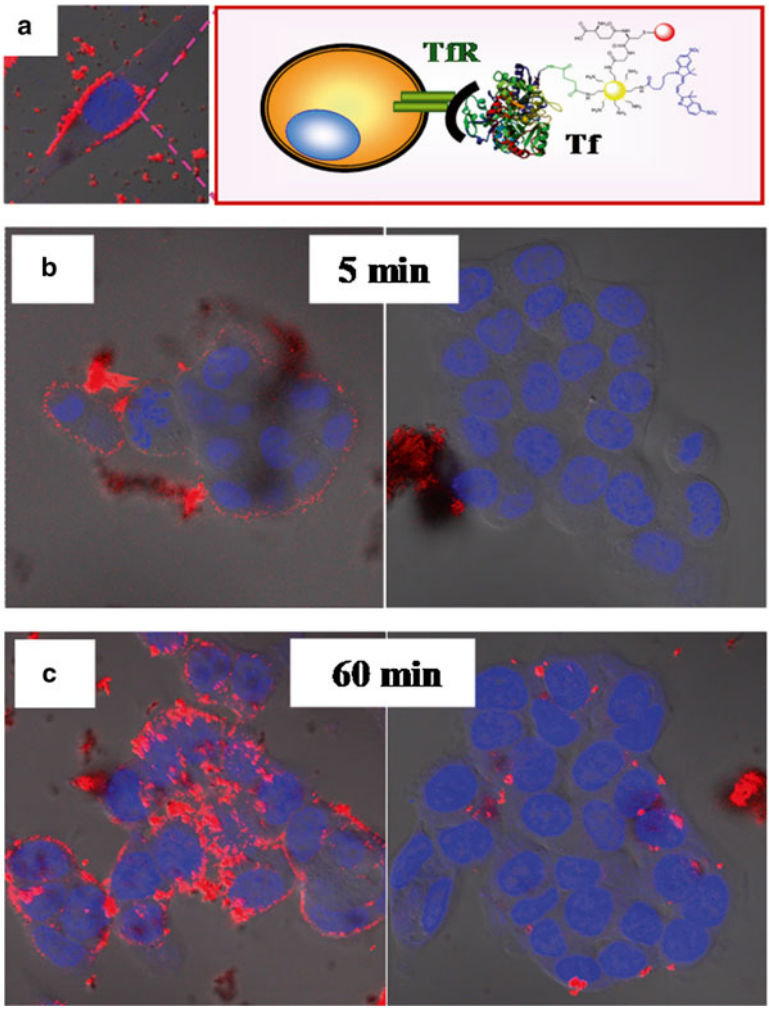

Fig. 11.7 Cellular targeting of a bio-functionalized Magneto-Dendritic Nano System (MDNS). (**a**) Magnified image of a cell showing localization of MDNS particles on the cell membrane after 5 min of incubation. (**b**) *Left image* shows Tf^{+} MDNS particles attached to the HCT116 cells, whereas, hardly any Tf^{-} MDNS particles present on the cell surface as shown in right image. (**c**) After 60 min of exposure only the Tf^{+} MDNS were present in large numbers on the cells revealing target specificity of MDNS interaction remains intact even after long exposure (reproduced from [65])

TfR-overexpressing (TfR^{+}) cancer cells at clinically relevant concentrations (approximately 1 CTC per 10^5 blood cells) [65].

Choi and his coworkers showed that Tf decorated PEGylated gold nanoparticles accumulations in the tumors and other organs are independent of Tf (Fig. 11.8) [74]. However, the nanoparticle localizations within a particular organ are influenced by

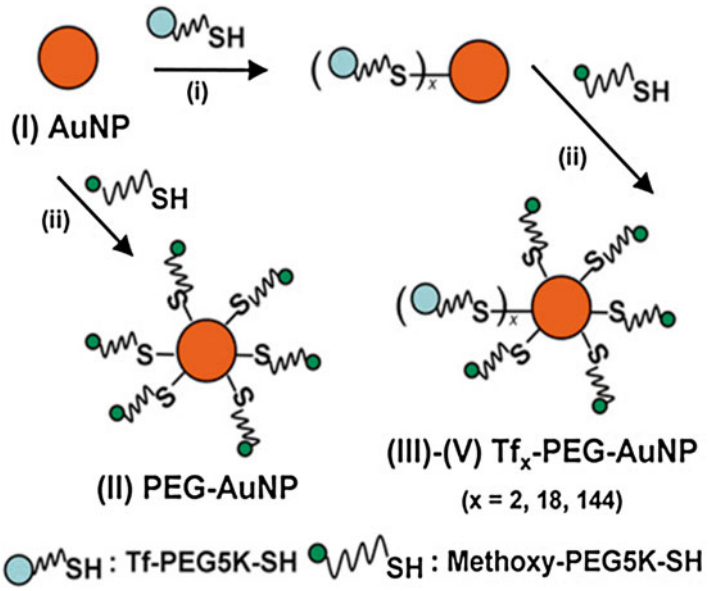

Fig. 11.8 Schematic of Tf-PEG-AuNPs. Unmodified 50-nm AuNPs (I) were reacted with excess mPEG-SH to form PEG-AuNPs (II) as untargeted particles or first were reacted with various amounts of Tf-PEG-SH and later excess mPEG-SH to form Tf-PEG-AuNPs (III: 2 Tf per particle; IV: 18 Tf per particle; V: 144 Tf per particle) (reproduced from [74])

the Tf content. They also demonstrated that in tumor tissue, the content of targeting ligands significantly influenced the number of nanoparticles localized within the cancer cells. Most nanoparticles remain in nonparenchymal cells, however, small amount of nanoparticles resided in hepatocytes due to higher Tf content [74].

Similarly, Lutenizing Hormone Releasing Hormone (LHRH) peptide-conjugated prodrug has shown promising results for cancer therapy [75, 8]. Khandare et al. conjugated LHRH peptide using PEG as the spacer to camptothecin (CPT), a cytotoxic drug [75, 8] (Fig. 11.9). The LHRH peptide-conjugated prodrug demonstrated higher efficacy with minimized side effects on healthy organs. In addition, the prodrug system showed targeting potential for both solid tumor tissue as well as a single tumor cell. Hence, this prodrug can effectively target tumor cells with a low toxicity to normal tissues [75]. Neamati and coworkers conjugated paclitaxel, an antimicrotubule agent commonly used in the treatment of metastatic breast cancer to a cyclic peptide E[c(RGDyK)]$_2$ (RGD). In vivo studies showed a specific tumor uptake of the RGD-paclitaxel system at 4 h post administration [76].

Similarly, Yoneda et al. have explored targeted delivery of paclitaxel and doxorubicin by using glucose-regulated protein 78 (GRP78) as a tumor-specific antigen [77]. GRP78 is overexpressed on many tumor cells, including skin, prostate, colon,

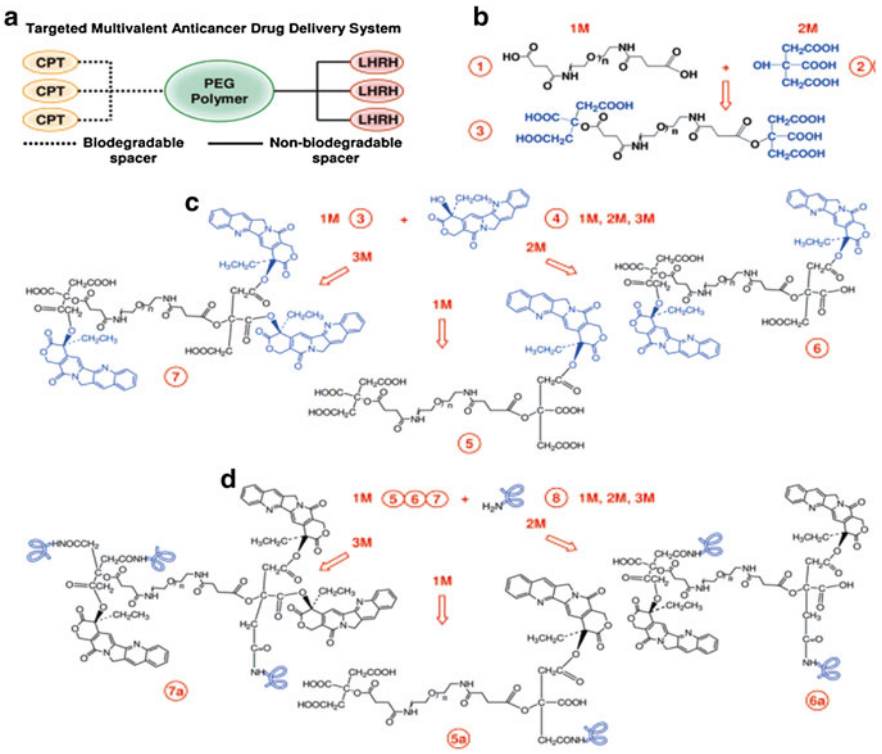

Fig. 11.9 Schematic of targeted multivalent anticancer prodrug. The prodrug conjugate system was designed with (**a**) bis-PEG polymer as a carrier- one, two, or three copies of CPT as an anticancer drug; and one, two, or three copies of LHRH peptide as a targeting agent. (**b**) α,ω-bis-PEG3000-CA conjugate (3) was designed by conjugation of bis(2-carboxyethyl) PEG (1) with CA (2). (**c**) The bis-PEG-CA conjugate (3) was conjugated with CPT (4) to obtain α,ω-bis(2-carboxyethyl) PEG-CA-CPT conjugates (5, 6, and 7). (**d**) α,ω-bis(2-carboxyethyl) PEG-CA-CPT-LHRH conjugates (d, 5a, 6a, and 7a) having one, two, and three copies of CPT (4) and LHRH (8) were synthesized by conjugating LHRH (8) with 5, 6, and 7 (reproduced from [75])

and breast cancers. On the other hand, its expression on normal tissues is very small [78]. Pep42, a cyclic 13-meroligopeptide (CTVALPGGYVRVC), internalizes through the GRP78 receptor-mediated endocytosis after specifically binding to GRP78 and then trafficked to the lysosome that contains protease cathepsin B. Therefore, Val-Cit motif, a cleavable linker, was used to link Pep42 to anticancer drugs. The Val-Cit linker is reasonably stable in the plasma, but cathepsin B in the cancer cells can cleave [79]. Both Pep42-paclitaxel and Pep42-Dox showed an enhanced toxicity in comparison to the free drug when cytotoxicity of the Pep42-prodrug was estimated in osteosarcoma cells, SJSA-1 [77].

11.7 Folic Acid-Drug Conjugate

Folic acid (FA) is a member of the vitamin B family and is one of the most commonly used targeting moiety for specific delivery of various imaging agents, therapeutic agents, and nano-scaled systems to tumor cells. It is known to bind with a very high affinity (K_d 0.1–1 nM) to folate receptor (FR). Folate receptor is overexpressed on the surface of many malignant cells including breast, lung, kidney, ovarian, and endometrial cancers [80]. On the other hand, the expression of FR on other normal tissues is low and restricted to some epithelial cells. Folic acid conjugated prodrugs enter cells via receptor mediated endocytosis after binding to folate receptors. In addition, FA has a low immunogenicity and relatively simple chemistry compared to other targeting moieties such as antibody, peptide, and aptamer [80–82]. For targeting tumor cells, a range of anticancer drugs have been conjugated with FA.

To enhance the specificity to tumor cells Dox was conjugated to FA [83]. Dox is an anthracyclinic drug used for a wide variety of cancers. However, poor solubility, extremely high toxicity and short half-life limit its therapeutic efficacy. D-α-Tocopheryl polyethylene glycol succinate (TPGS) was conjugated, to the FA modification, to Dox to enhance the solubility and drug permeability across cell membrane. The TPGS-Dox-FA prodrug exhibited enhanced half-life, high antitumor efficacy (45-fold more effective than the unmodified Dox), and less accumulation in the heart, which is the major organ affected by Dox's side effects [83].

Philip et al. demonstrated that FA on conjugation to campothecin, a poor water-soluble and highly toxic chemotherapy agent, via a hydrophilic peptide containing a disulfide bond [81]. They showed increase in specificity of the prodrug, while the cleavable spacer increased the solubility of campothecin and also provides an efficient release of camptothecin within tumor cells via the disulfide reduction [81].

11.8 Conclusions

Polymeric prodrug conjugate systems offer a potent and versatile tool for improving the therapeutic potential of low-molecular-weight drugs and proteins. Although considerable progress has been made in the field of prodrugs in clinic, prodrug systems consisting a targeting component still remains only as a perspective. There are numerous scientific challenges to overcome this goal, such as advanced biomaterials, molecular tunability, physicochemical strategies, immunogenicity, cell permeability, and cell specificity in clinically relevant targeted drugs. However, several prodrug systems having anticancer agents are currently under clinical trials. Towards this direction, in next few years many other anticancer conjugates could get regulatory approvals. In the future, requirement for advance drug delivery strategies such as targeted prodrugs will become more important since the discovery of new anticancer drugs will become increasingly challenging and expensive [1].

References

1. Knox RJ, Connors TA (1997) Prodrugs in cancer chemotherapy. Pathol Oncol Res 3(4):309–324
2. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, Savolainen J (2008) Prodrugs: design and clinical applications. Nat Rev Drug Discov 7(3):255–270. doi:10.1038/nrd2468
3. Duncan R (2003) The dawning era of polymer therapeutics. Nat Rev Drug Discov 2(5):347–360. doi:10.1038/nrd1088
4. Singh Y, Palombo M, Sinko PJ (2008) Recent trends in targeted anticancer prodrug and conjugate design. Curr Med Chem 15(18):1802–1826
5. Muller CE (2009) Prodrug approaches for enhancing the bioavailability of drugs with low solubility. Chem Biodivers 6(11):2071–2083. doi:10.1002/cbdv.200900114
6. Minko T, Khandare JJ, Vetcher AA, Soldatenkov CA, Garbuzenko OB, Saad M, Pozharov VP (2008) Multifunctional nanotherapeutics for cancer. In: Torchilin VP (ed) Multifunctional pharmaceutical nanocarriers. Springer, New York, pp 309–336
7. Minko T (2004) Drug targeting to the colon with lectins and neoglycoconjugates. Adv Drug Deliv Rev 56(4):491–509. doi:10.1016/j.addr.2003.10.017
8. Dharap SS, Wang Y, Chandna P, Khandare JJ, Qiu B, Gunaseelan S, Sinko PJ, Stein S, Farmanfarmaian A, Minko T (2005) Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide. Proc Natl Acad Sci U S A 102(36):12962–12967. doi:10.1073/pnas.0504274102
9. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 65(1–2):271–284
10. Mariano WA, Stella VJ (2007) Macromolecular prodrugs of small molecules. In: Stella V, Borchardt R, Hageman M, Oliyai R, Maag H, Tilley J (eds) Prodrugs, challenges and rewards part 1, vol 1. Springer-AAPS, New York, pp 289–321. doi:10.1007/978-0-387-49785-3
11. Greish K (2007) Enhanced permeability and retention of macromolecular drugs in solid tumors: a royal gate for targeted anticancer nanomedicines. J Drug Target 15(7–8):457–464. doi:10.1080/10611860701539584
12. Haag R, Kratz F (2006) Polymer therapeutics: concepts and applications. Angew Chem Int Ed Engl 45(8):1198–1215. doi:10.1002/anie.200502113
13. Jain RK (1994) Barriers to drug delivery in solid tumors. Sci Am 271(1):58–65
14. Minko T, Dharap SS, Pakunlu RI, Wang Y (2004) Molecular targeting of drug delivery systems to cancer. Curr Drug Targets 5(4):389–406
15. Choksi A, Sarojini KV, Vadnal P, Dias C, Suresh PK, Khandare J (2013) Comparative anti-inflammatory activity of poly(amidoamine) (PAMAM) dendrimer-dexamethasone conjugates with dexamethasone-liposomes. Int J Pharm 449(1–2):28–36. doi:10.1016/j.ijpharm.2013.03.056
16. Banerjee SS, Aher N, Patil R, Khandare J (2012) Poly(ethylene glycol)-prodrug conjugates: concept, design, and applications. J Drug Deliv 2012:103973. doi:10.1155/2012/103973
17. Maison W, Frangioni JV (2003) Improved chemical strategies for the targeted therapy of cancer. Angew Chem Int Ed 42(39):4726–4728. doi:10.1002/anie.200301666
18. Khandare J, Minko T (2006) Polymer–drug conjugates: progress in polymeric prodrugs. Prog Polym Sci 31(4):359–397. doi:http://dx.doi.org/10.1016/j.progpolymsci.2005.09.004
19. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2(12):751–760. doi:10.1038/nnano.2007.387
20. Administration USFDA (2014) Hematology/Oncology (Cancer) Approvals & Safety Notifications. http://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm279174.htm. Accessed 23 March 2014
21. Collins I, Workman P (2006) New approaches to molecular cancer therapeutics. Nat Chem Biol 2(12):689–700. doi:10.1038/nchembio840

22. Park JW, Hong K, Kirpotin DB, Meyer O, Papahadjopoulos D, Benz CC (1997) Anti-HER2 immunoliposomes for targeted therapy of human tumors. Cancer Lett 118(2):153–160
23. Baselga J (2001) The EGFR as a target for anticancer therapy—focus on cetuximab. Eur J Cancer 37(Suppl 4):S16–S22
24. Fischer PM, Gianella-Borradori A (2003) CDK inhibitors in clinical development for the treatment of cancer. Expert Opin Investig Drugs 12(6):955–970. doi:10.1517/13543784.12.6.955
25. Ellis LM, Hicklin DJ (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer 8(8):579–591. doi:10.1038/nrc2403
26. Dietel M, Johrens K, Laffert M, Hummel M, Blaker H, Muller BM, Lehmann A, Denkert C, Heppner FL, Koch A, Sers C, Anagnostopoulos I (2013) Predictive molecular pathology and its role in targeted cancer therapy: a review focussing on clinical relevance. Cancer Gene Ther 20(4):211–221. doi:10.1038/cgt.2013.13
27. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD (2008) Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26(10):1626–1634. doi:10.1200/jco.2007.14.7116
28. Gilcrease MZ, Woodward WA, Nicolas MM, Corley LJ, Fuller GN, Esteva FJ, Tucker SL, Buchholz TA (2009) Even low-level HER2 expression may be associated with worse outcome in node-positive breast cancer. Am J Surg Pathol 33(5):759–767. doi:10.1097/PAS.0b013e31819437f9
29. Hudziak RM, Schlessinger J, Ullrich A (1987) Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells. Proc Natl Acad Sci U S A 84(20):7159–7163
30. Hudis CA (2007) Trastuzumab—mechanism of action and use in clinical practice. N Engl J Med 357(1):39–51. doi:10.1056/NEJMra043186
31. Morrow PK, Wulf GM, Ensor J, Booser DJ, Moore JA, Flores PR, Xiong Y, Zhang S, Krop IE, Winer EP, Kindelberger DW, Coviello J, Sahin AA, Nunez R, Hortobagyi GN, Yu D, Esteva FJ (2011) Phase I/II study of trastuzumab in combination with everolimus (RAD001) in patients with HER2-overexpressing metastatic breast cancer who progressed on trastuzumab-based therapy. J Clin Oncol 29(23):3126–3132. doi:10.1200/jco.2010.32.2321
32. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2(7):489–501. doi:10.1038/nrc839
33. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, van de Vijver MJ, Bernards R (2007) A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell 12(4):395–402. doi:10.1016/j.ccr.2007.08.030
34. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT, Hortobagyi GN, Hung MC, Yu D (2004) PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 6(2):117–127. doi:10.1016/j.ccr.2004.06.022
35. Damodaran S, Olson EM (2012) Targeting the human epidermal growth factor receptor 2 pathway in breast cancer. Hosp Pract 40(4):7–15. doi:10.3810/hp.2012.10.997
36. Jerusalem G, Fasolo A, Dieras V, Cardoso F, Bergh J, Vittori L, Zhang Y, Massacesi C, Sahmoud T, Gianni L (2011) Phase I trial of oral mTOR inhibitor everolimus in combination with trastuzumab and vinorelbine in pre-treated patients with HER2-overexpressing metastatic breast cancer. Breast Cancer Res Treat 125(2):447–455. doi:10.1007/s10549-010-1260-x
37. Bull C, Boltje TJ, Wassink M, de Graaf AM, van Delft FL, den Brok MH, Adema GJ (2013) Targeting aberrant sialylation in cancer cells using a fluorinated sialic acid analog impairs adhesion, migration, and in vivo tumor growth. Mol Cancer Ther 12(10):1935–1946. doi:10.1158/1535-7163.mct-13-0279
38. Khandare JJ, Wang Y, Singh AP, Vorsa N, Minko T (2007) Targeted sialic acid-doxorubicin prodrugs for intracellular delivery and cancer treatment. Pharm Res 24(11):2120–2130. doi:10.1007/s11095-007-9406-1

39. Cheng WW, Allen TM (2008) Targeted delivery of anti-CD19 liposomal doxorubicin in B-cell lymphoma: a comparison of whole monoclonal antibody, Fab' fragments and single chain Fv. J Control Release 126(1):50–58. doi:10.1016/j.jconrel.2007.11.005
40. Sapra P, Moase EH, Ma J, Allen TM (2004) Improved therapeutic responses in a xenograft model of human B lymphoma (Namalwa) for liposomal vincristine versus liposomal doxorubicin targeted via anti-CD19 IgG2a or Fab' fragments. Clin Cancer Res 10(3):1100–1111
41. Chapman AP (2002) PEGylated antibodies and antibody fragments for improved therapy: a review. Adv Drug Deliv Rev 54(4):531–545
42. Maloney DG, Grillo-Lopez AJ, White CA, Bodkin D, Schilder RJ, Neidhart JA, Janakiraman N, Foon KA, Liles TM, Dallaire BK, Wey K, Royston I, Davis T, Levy R (1997) IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. Blood 90(6):2188–2195
43. Chan SY, Gordon AN, Coleman RE, Hall JB, Berger MS, Sherman ML, Eten CB, Finkler NJ (2003) A phase 2 study of the cytotoxic immunoconjugate CMB-401 (hCTM01-calicheamicin) in patients with platinum-sensitive recurrent epithelial ovarian carcinoma. Cancer Immunol Immunother 52(4):243–248. doi:10.1007/s00262-002-0343-x
44. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D, Mountain A, Flowers DA, Bernstein I (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconjug Chem 13(1):47–58
45. Tai W, Mahato R, Cheng K (2010) The role of HER2 in cancer therapy and targeted drug delivery. J Control Release 146(3):264–275. doi:10.1016/j.jconrel.2010.04.009
46. Marasco D, Perretta G, Sabatella M, Ruvo M (2008) Past and future perspectives of synthetic peptide libraries. Curr Protein Pept Sci 9(5):447–467
47. Koivunen E, Arap W, Rajotte D, Lahdenranta J, Pasqualini R (1999) Identification of receptor ligands with phage display peptide libraries. J Nucl Med 40(5):883–888
48. Smith GP, Petrenko VA (1997) Phage display. Chem Rev 97(2):391–410
49. Brown KC (2000) New approaches for cell-specific targeting: identification of cell-selective peptides from combinatorial libraries. Curr Opin Chem Biol 4(1):16–21
50. Pasqualini R, Koivunen E, Ruoslahti E (1997) Alpha v integrins as receptors for tumor targeting by circulating ligands. Nat Biotechnol 15(6):542–546. doi:10.1038/nbt0697-542
51. Koivunen E, Wang B, Ruoslahti E (1995) Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. Biotechnology (N Y) 13(3):265–270
52. Doorbar J, Winter G (1994) Isolation of a peptide antagonist to the thrombin receptor using phage display. J Mol Biol 244(4):361–369. doi:10.1006/jmbi.1994.1736
53. Oyama T, Sykes KF, Samli KN, Minna JD, Johnston SA, Brown KC (2003) Isolation of lung tumor specific peptides from a random peptide library: generation of diagnostic and cell-targeting reagents. Cancer Lett 202(2):219–230
54. Oyama T, Rombel IT, Samli KN, Zhou X, Brown KC (2006) Isolation of multiple cell-binding ligands from different phage displayed-peptide libraries. Biosens Bioelectron 21(10):1867–1875. doi:10.1016/j.bios.2005.11.016
55. McGuire MJ, Samli KN, Chang YC, Brown KC (2006) Novel ligands for cancer diagnosis: selection of peptide ligands for identification and isolation of B-cell lymphomas. Exp Hematol 34(4):443–452. doi:10.1016/j.exphem.2005.12.013
56. McGuire MJ, Samli KN, Johnston SA, Brown KC (2004) In vitro selection of a peptide with high selectivity for cardiomyocytes in vivo. J Mol Biol 342(1):171–182. doi:10.1016/j.jmb.2004.06.029
57. Samli KN, McGuire MJ, Newgard CB, Johnston SA, Brown KC (2005) Peptide-mediated targeting of the islets of Langerhans. Diabetes 54(7):2103–2108
58. Baines IC, Colas P (2006) Peptide aptamers as guides for small-molecule drug discovery. Drug Discov Today 11(7–8):334–341. doi:10.1016/j.drudis.2006.02.007
59. Jabbari E (2009) Targeted delivery with peptidomimetic conjugated self-assembled nanoparticles. Pharm Res 26(3):612–630. doi:10.1007/s11095-008-9802-1

60. Curnis F, Gasparri A, Sacchi A, Longhi R, Corti A (2004) Coupling tumor necrosis factor-alpha with alphaV integrin ligands improves its antineoplastic activity. Cancer Res 64(2):565–571
61. Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Rio GD, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE, Pasqualini R (1999) Anti-cancer activity of targeted pro-apoptotic peptides. Nat Med 5(9):1032–1038. doi:10.1038/12469
62. Laakkonen P, Akerman ME, Biliran H, Yang M, Ferrer F, Karpanen T, Hoffman RM, Ruoslahti E (2004) Antitumor activity of a homing peptide that targets tumor lymphatics and tumor cells. Proc Natl Acad Sci U S A 101(25):9381–9386. doi:10.1073/pnas.0403317101
63. Laakkonen P, Porkka K, Hoffman JA, Ruoslahti E (2002) A tumor-homing peptide with a targeting specificity related to lymphatic vessels. Nat Med 8(7):751–755. doi:10.1038/nm720
64. Zhang L, Giraudo E, Hoffman JA, Hanahan D, Ruoslahti E (2006) Lymphatic zip codes in premalignant lesions and tumors. Cancer Res 66(11):5696–5706. doi:10.1158/0008-5472.can-05-3876
65. Banerjee SS, Jalota-Badhwar A, Satavalekar SD, Bhansali SG, Aher ND, Mascarenhas RR, Paul D, Sharma S, Khandare JJ (2013) Transferrin-mediated rapid targeting, isolation, and detection of circulating tumor cells by multifunctional magneto-dendritic nanosystem. Adv Healthc Mater 2(6):800–805. doi:10.1002/adhm.201200164
66. Faulk WP, Taylor CG, Yeh CJ, McIntyre JA (1990) Preliminary clinical study of transferrin-adriamycin conjugate for drug delivery to acute leukemia patients. Mol Biother 2(1):57–60
67. Head JF, Wang F, Elliott RL (1997) Antineoplastic drugs that interfere with iron metabolism in cancer cells. Adv Enzyme Regul 37:147–169
68. Rainov NG, Soling A (2005) Technology evaluation: transMID, KS Biomedix/Nycomed/Sosei/PharmaEngine. Curr Opin Mol Ther 7(5):483–492
69. Bellocq NC, Pun SH, Jensen GS, Davis ME (2003) Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. Bioconjug Chem 14(6):1122–1132. doi:10.1021/bc034125f
70. Heidel JD, Yu Z, Liu JY, Rele SM, Liang Y, Zeidan RK, Kornbrust DJ, Davis ME (2007) Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. Proc Natl Acad Sci U S A 104(14):5715–5721. doi:10.1073/pnas.0701458104
71. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ (2005) Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. Cancer Res 65(19):8984–8992. doi:10.1158/0008-5472.can-05-0565
72. Pun SH, Tack F, Bellocq NC, Cheng J, Grubbs BH, Jensen GS, Davis ME, Brewster M, Janicot M, Janssens B, Floren W, Bakker A (2004) Targeted delivery of RNA-cleaving DNA enzyme (DNAzyme) to tumor tissue by transferrin-modified, cyclodextrin-based particles. Cancer Biol Ther 3(7):641–650
73. Wu J, Lu Y, Lee A, Pan X, Yang X, Zhao X, Lee RJ (2007) Reversal of multidrug resistance by transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil. J Pharm Pharm Sci 10(3):350–357
74. Choi CH, Alabi CA, Webster P, Davis ME (2010) Mechanism of active targeting in solid tumors with transferrin-containing gold nanoparticles. Proc Natl Acad Sci U S A 107(3):1235–1240. doi:10.1073/pnas.0914140107
75. Khandare JJ, Chandna P, Wang Y, Pozharov VP, Minko T (2006) Novel polymeric prodrug with multivalent components for cancer therapy. J Pharmacol Exp Ther 317(3):929–937. doi:10.1124/jpet.105.098855
76. Chen X, Plasencia C, Hou Y, Neamati N (2005) Synthesis and biological evaluation of dimeric RGD peptide-paclitaxel conjugate as a model for integrin-targeted drug delivery. J Med Chem 48(4):1098–1106. doi:10.1021/jm049165z
77. Yoneda Y, Steiniger SC, Capkova K, Mee JM, Liu Y, Kaufmann GF, Janda KD (2008) A cell-penetrating peptidic GRP78 ligand for tumor cell-specific prodrug therapy. Bioorg Med Chem Lett 18(5):1632–1636. doi:10.1016/j.bmcl.2008.01.060

78. Shin BK, Wang H, Yim AM, Le Naour F, Brichory F, Jang JH, Zhao R, Puravs E, Tra J, Michael CW, Misek DE, Hanash SM (2003) Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. J Biol Chem 278(9):7607–7616. doi:10.1074/jbc.M210455200
79. Dubowchik GM, Firestone RA (1998) Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. Bioorg Med Chem Lett 8(23):3341–3346
80. Shangguan D, Li Y, Tang Z, Cao ZC, Chen HW, Mallikaratchy P, Sefah K, Yang CJ, Tan W (2006) Aptamers evolved from live cells as effective molecular probes for cancer study. Proc Natl Acad Sci U S A 103(32):11838–11843. doi:10.1073/pnas.0602615103
81. Henne WA, Doorneweerd DD, Hilgenbrink AR, Kularatne SA, Low PS (2006) Synthesis and activity of a folate peptide camptothecin prodrug. Bioorg Med Chem Lett 16(20):5350–5355. doi:10.1016/j.bmcl.2006.07.076
82. Aronov O, Horowitz AT, Gabizon A, Gibson D (2003) Folate-targeted PEG as a potential carrier for carboplatin analogs. Synthesis and in vitro studies. Bioconjug Chem 14(3):563–574. doi:10.1021/bc025642l
83. Anbharasi V, Cao N, Feng SS (2010) Doxorubicin conjugated to D-alpha-tocopheryl polyethylene glycol succinate and folic acid as a prodrug for targeted chemotherapy. J Biomed Mater Res A 94(3):730–743. doi:10.1002/jbm.a.32734

Chapter 12
Polymer–Drug Conjugates for Targeted Drug Delivery

Anjan Kumar Mohanty, Fahima Dilnawaz, Guru Prasad Mohanta, and Sanjeeb Kumar Sahoo

Abbreviations

Ara-C	1-b-D-Arabinofuranosylcytosine
CPT	Camptothecin
DDS	Drug delivery system
DOX	Doxorubicin
EPR	Enhanced permeability and retention
HPMA	*N*-(2-Hydroxypropyl) methacrylamide
MA	Methacrylate
MAP	Mucic acid polymer
MDR	Multidrug resistance
PEG	Poly(ethylene glycol)
PG	Poly-L-glutamic acid
RES	Reticuloendothelial system
SMA	Styrene maleic anhydride
TADD	Tissue Activated Drug Delivery
TXL	Paclitaxel

A.K. Mohanty • G.P. Mohanta
Department of Pharmacy, Annamalai University,
Annamalai Nagar, Chidambaram, Tamilnadu, India

F. Dilnawaz • S.K. Sahoo, Ph.D. (✉)
Laboratory of Nanomedicine, Institute of Life Sciences,
Nalco Square, Chandrasekharpur, Bhubaneswar 751023, Odisha, India
e-mail: sanjeebsahoo2005@gmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_12

12.1 Drug Delivery Challenges and Pharmacokinetics of Polymer–Drug Conjugates

A drug delivery system (DDS) is defined as a formulation or a device that enables the administration of therapeutic substances in the body and improves its efficacy and safety. Over the past three decades, significant advances have been made in drug delivery technology. Modern technological approach, such as nanotechnology based drug delivery system has profound impact on disease prevention, diagnosis, and treatment in terms of its stability, absorption, therapeutic concentration, and improved pharmacokinetics over native drugs [1–3]. In order to improve the specific delivery of drugs with a low therapeutic index, several nanosized drug carriers have been developed. These nanoformulation vehicles are the polymeric nanoparticles, amphiphilic core/shell (polymeric micelles or liposomes) or hyperbranched macromolecules (dendrimers, drug–polymer conjugates), etc. [4–9].

The concepts of utilizing polymers as therapeutic agents have been widely investigated for a number of decades. In 1975, Helmut Ringsdorf proposed the concept of polymer therapeutics, where a polymer is covalently bound to drug molecules that could improve the aqueous solubility and bioavailability of the drug [10]. These polymer–drug conjugates offers several significant advantages over traditional small molecule therapeutics as they can protect it from degradation, resulting in improved efficacy due to increased drug circulation times, controlled release of drugs in terms of variations in pH, temperature, enzyme concentration, or attachment of ligands for targeting to the desired site of therapeutic need [11, 12]. Further, the polymer–drug conjugates increases the reduction in the uptake by reticuloendothelial system (RES) or macrophages due to stealth effect of the polymer [12]. The increased blood circulation time of the polymer–drug conjugate also enhances the therapeutic index of the drug in tumor tissues, by taking the advantages of leaky vasculature and impaired lymphatic drainage system known as enhanced permeability and retention (EPR) effect (Fig. 12.1) [13–15].

12.2 Important Aspects of Design of Polymer–Drug Conjugates

The basic requirements for the design of polymer–drug conjugates are based on the worldwide screening of the natural and synthetic polymers and its use in vivo screening to find out a biocompatible polymer (i.e., nontoxic, nonimmunogenic, biodegradable, etc.). The identification of the new pharmacological targets arises from the molecular basis of the diseases. A wide range of polymers are available for the delivery of macromolecular drugs [16]. These macromolecular prodrugs comprise a minimum of three components: (1) a natural or synthetic water soluble polymeric carriers; (2) a biodegradable polymer–drug linker; and (3) a bioactive

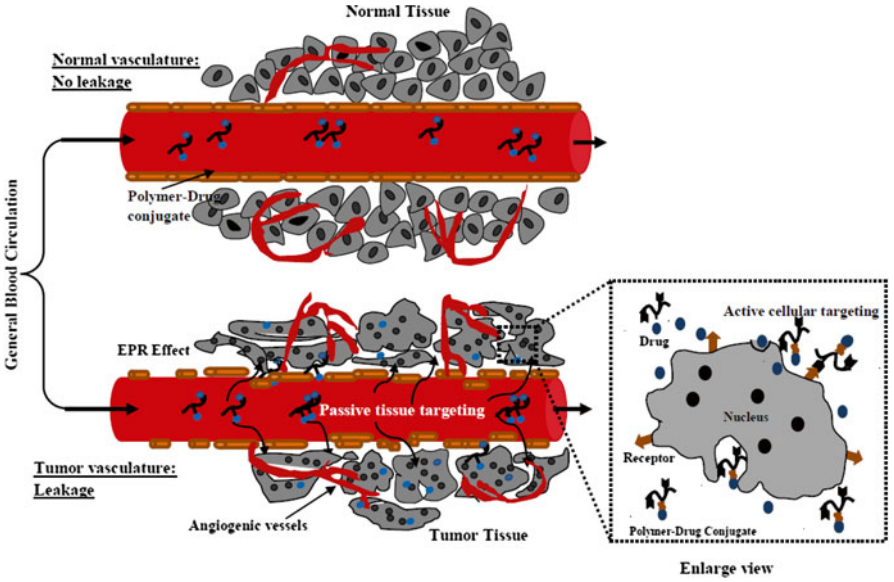

Fig. 12.1 Schematic representative of EPR-mediated tumor targeting

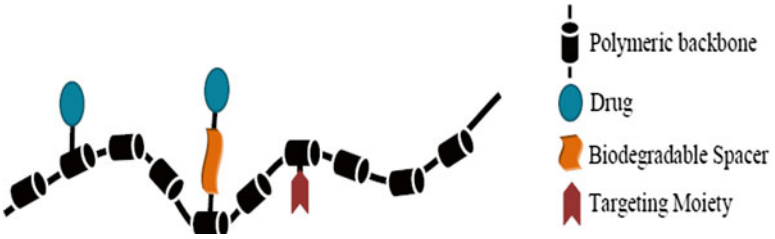

Fig. 12.2 Schematic presentation of polymer–drug conjugates in which a solubility enhancer, a targeting moiety, a spacer, and specific drugs could be attached to the same polymeric chain

antitumor agent (Fig. 12.2). Ligands can also be attached to facilitate active targeting. An ideal targeting system should enable it to overcome the biological barriers that selectively direct it against the diseased cells [17, 18].

12.2.1 The Polymer

The different polymers that are used for the drug conjugation includes *N*-(2-hydroxypropyl) methacrylamide (HPMA), poly-L-glutamic acid (PG), polyethylene glycol (PEG), styrene maleic anhydride (SMA), etc. [19, 20]. These polymers also have been exploited for conjugation to various proteins and drugs. Currently considerable research effort is being focused on the continued development of

improved drug delivery systems. Mostly the polymer–drug conjugate systems are designed for localization of the chemotherapeutic drugs in the targeted mainly cancer tissues to provide a sustained release of drug activity over weeks to months [21]. For this, the choice of polymers should contain the required functional group for the covalent linkage of the drug. Apart from that, the drug carriers for the parenteral administration should have the property of biodegradability, hydrophilicity, and the ability to carry the required payload of the drug for an effective therapeutic application [22].

12.2.2 The Polymer–Drug Linker

In the preparation of the polymer–drug conjugates a biodegradable spacer/linker is inserted to ensure stability in the circulation and subsequently facilitate specific enzymatic or hydrolytic intratumoral drug release. The polymer–drug linker also plays an important role in keeping inactive prodrug until released from the backbone polymer by a disease-specific trigger to help in enhancing the circulatory half-life and biodistribution of the drug [23, 24]. This characteristic feature of disease-specific release from the polymer conjugate has been coined as Tissue Activated Drug Delivery (TADD). Theoretically, the disease specific trigger alters the biodistribution of the active agent, releasing it near the site of pathology. The hydrolytic or enzymatic cleavage of the linker in drug–polymer conjugate is also helpful in the release of the drug, for example presence of the esterases breaks the ester linkage of the conjugation, while amide linkage gives stability in the blood circulation. In some cases the systemic delivery of the drug can be achieved by designing sensitive linkers effective at pH 7.2. Therefore, the choosing desirable option of the linker will be such that it should be stable in the circulation and protect the drug against premature metabolism but amenable to specific enzymatic or hydrolytic cleavage intratumorally.

12.2.3 Ligands

The ligands are used to increase the receptor mediated binding and internalisation of the therapeutic agent into the cells of target tissues. Tumor cells specifically overexpress particular cell surface marker as compared to normal cells, which helps in active targeting and increased cellular uptake. These molecular markers specifically help in active targeting and increased cellular uptake in tumor tissues. Looking at the specificity of the overexpressed markers, the conjugating ligand can be antibody, antibody fragment, peptides, saccharides, or other small molecules. Other targeting moieties such as peptides, sugars, and hormones can generally be readily synthesized at low cost, but they typically have reduced binding affinity and specificity as compared to antibodies and antibody fragments. Ray and coworkers

developed the HPMA copolymers containing cyclic Arg-Gly-Asp (RGD) peptides that target αvβ3 integrins expressed on angiogenic tumor cells and found that the HPMA copolymer–aminohexylgeldanamycin–cyclic RGD had more efficacy via active targeting as compared to conjugates relying solely on "passive" targeting via the EPR effect [25]. Tang et al. used epitope containing HPMA copolymer–doxorubicin conjugates to target the CD21+ Raji B-cells and CD21− HSB-2T-cells. The results showed that, epitope-HPMA copolymer–doxorubicin conjugate have more biorecognition activity towards the Raji cells which demonstrated enhanced cytotoxicity than the free doxorubicin [26].

12.3 Polymer–Drug Conjugates as Drug Delivery Systems

In polymer–drug conjugates the drug is covalently attached to the polymeric backbone. The structure and architecture of the polymer gives scope for designing novel polymer–drug conjugates for drug delivery. Over the past few years, a number of polymer–drug conjugates have entered clinical studies (Table 12.1). In this regard potential therapeutic application of different polymer–drug conjugates are discussed with special emphasis on HPMA, PG and PEG (Fig. 12.3).

12.3.1 N-*(2-Hydroxypropyl)methacrylamide (HPMA) Copolymers*

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer belongs to homopolymer group. It has the characteristic features of highly water solubility, non-immunogenic, nontoxic, non-binding activity to blood proteins with long biological half-life. Thus, it can be used as macromolecular carrier for the low molecular weight anticancer chemotherapeutic to amplify the circulation time of the therapeutic agent in blood and limit the side effects. The HPMA copolymer is generally synthesised by free radical polymerization using HPMA and methacrylated (MA)-peptidyl-nitrophenylester (ONp) as co-monomers in a ratio of 95:5 % w/w. The synthesis of narrow molecular weight HPMA copolymer (Mw/Mn = 1.2–1.5 kDa) is possible by careful control of the co-monomers and reaction conditions for polymerization kinetics. The HPMA copolymer backbone has the disadvantage that it is not inherently biodegradable. So there is risk of cellular accumulation due to sequestration in the lysosomal compartment, however the conjugates with molecular weight of <40,000 g/mol could ensure elimination by glomerular filtration after parenteral administration. To overcome these problems the back bone of the HPMA copolymer can be grafted by various functionalized co-monomers, allowing control over the composition of these conjugate systems and reduce the immunogenicity. Kopecek et al. investigated the biocompatibility properties of the HPMA polymer by substituting the α-carbon and the presence of an amide linkage in the side chain to ensure hydrolytic stability [20, 27].

Table 12.1 Polymer–drug conjugates in clinical trials

Conjugates	Indication	Status	Company
N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers–drug conjugate			
HPMA copolymer–doxorubicin (PK1; FCE28068)	Particularly lung and breast cancer	Phase II	Pfizer; CRC UK
HPMA copolymer–doxorubicin–galactosamine (PK2; FCE28069)	Hepatocellular carcinoma	Phase I/II	Pfizer; CRC UK
HPMA copolymer–platinate (AP5280, ProLindac)	Various cancers, particularly ovarian, colorectal cancers	Phase I/II	Access pharmaceutical
HPMA copolymer–DACH–platinate (AP5346, ProLindac)	Various cancers	Phase II	Access pharmaceutical
HPMA copolymer–paclitaxel (PNU166945)	Various solid tumors	Phase I; discontinued	Pfizer; CRC UK
HPMA copolymer–Camptothecin (PCNU166148)	Solid tumors	Phase I; discontinued	Pfizer Inc.
HPMA copolymer–carboplatinate (AP5280)	Ovarian carcinoma, lung and head cancers	Phase I/II	
Poly(α,L-glutamic acid)–drug conjugates			
PG–paclitaxel (CT-2103, Xyotax)	Lung, ovarian, colorectal, breast cancer	Phase III	Cell Therapeutics
PG–camptothecin (CT2106)	Colorectal, lung, and ovarian cancers	Phase I	Cell Therapeutics
Poly(ethylene glycol)–drug conjugates			
PEG–Camptothecin (Pegamotecan)	Solid tumors	Phase I; discontinued	Enzon Pharmaceuticals
PEG–SN38 (EZN-2208)	Solid tumors	Phase I	Enzon Pharmaceuticals
Other polymer–drug conjugates			
Carboxymethyl Dextran–camptothecin (IT-101)	Solid tumors	Phase I	Insert Therapeutics
Carboxymethyldextran–exatecan (DE-310)	Solid tumors	Phase I	Daiichi Pharmaceuticals

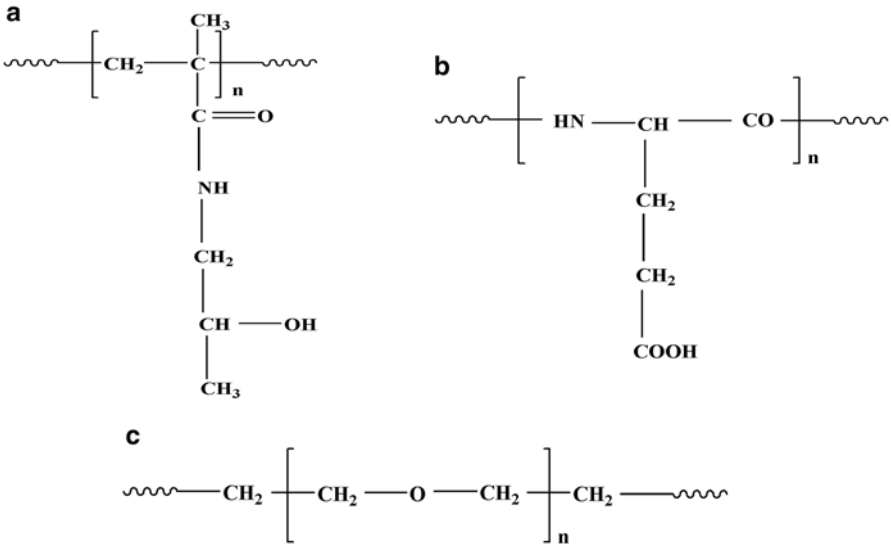

Fig. 12.3 Chemical structures of (**a**) *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers, (**b**) poly(α,L-glutamic acid) polymers, (**c**) poly(ethylene glycol) polymers

12.3.1.1 HPMA Copolymer–Doxorubicin

Doxorubicin is an anthracycline antibiotics used in cancer chemotherapy and works by intercalating with DNA along, with the most adverse effect of cardiac toxicity. It is commonly used in the treatment of hematological malignancies, carcinoma, and soft tissue sarcomas. To reduce the toxicity of the doxorubicin, it conjugated with water soluble HPMA copolymer. Malugin and coworkers has developed HPMA-copolymer–doxorubicin conjugate and evaluated the mechanism of apoptosis on the A2780 human ovarian carcinoma cells. The HPMA copolymer–doxorubicin conjugate acts both by the caspase-dependent and caspase-independent pathways of DNA damage and illustrated the enhanced level of apoptosis as compared to free doxorubicin [28]. The promising results of the in vitro study gave scope for the researchers to work further on the HPMA copolymer–doxorubicin conjugate. Seymour et al. reported that, the melanoma tumor bearing mice treated with HPMA copolymer–doxorubicin conjugate (PK1) accumulated ~65-fold of the increased doxorubicin as compared to free doxorubicin [9]. Again the stability of the PK1 was increased by the biodegradable tetrapeptide sequence GFLG (glycylphenylalanylleucylglycine) linkage. The GFLG linkage was designed for cleavage by lysosomal cathepsins, and also helpful in intravenous administration of the drug, which minimizes cardiotoxicity and bone marrow toxicity. Further, phase I clinical trials showed that, the effect of PK1 was marginal with 2 partial and 2 minor responses out of 36 chemotherapy-refractory patients. And in phase II study with patients having breast ($n = 17$), non-small-cell lung ($n = 29$), and colorectal ($n = 16$) cancer. Up to eight courses of PK1

(280 mg/m^2 doxorubicin-equivalent) were given by i.v. administration. Toxicities were tolerable, with grade 3 neutropenia more prominent in patients with breast cancer. Out of 14 anthracycline-naive patients with breast cancer 3 had partial responses. In 26 evaluated patients with non-small-cell lung cancer, 3 chemotherapy-naive patients had partial responses. In contrast, none of the 16 evaluated patients with colorectal cancer responded well [29]. These results supported the concept that polymer–drug conjugates improved anticancer activities of the native drugs.

12.3.1.2 HPMA Copolymer–Doxorubicin–Galactosamine

The galactosamine, a hexosamine derived from galactose is used as promising targeting agent for the hepatocyte asialoglycoprotein receptor. Galactosamine was bound to the HPMA copolymer backbone via a Gly-(D, L)Phe-Leu-Gly linker, which provides hydrophobicity to the HPMA copolymer chain due to the increased content of relative hydrophobic side chains. Seymour and coworkers developed HPMA copolymer–doxorubicin conjugate with the ligand ~27 kDa, doxorubicin content of ~5 wt% and galactosamine content of 1.5–2.5 mol%. For the distribution study they use I^{123} labelled HPMA copolymer–doxorubicin–galactosamine and used gamma camera imaging analogue to follow distribution. The phase I clinical trial conducted in 31 patients, where 23 patients have hepatocellular carcinoma showed a measurable partial response. Again the SPECT gamma camera imaging study indicated that the HPMA copolymer–doxorubicin–galactosamine concentration in liver was of 15–20 % of administered dose at 24 h with lower accumulation in hepatic tumor (3.2 %).With the progress of hepatoma the target asialoglycoprotein receptor is shed out. But still the doxorubicin concentration in hepatoma was 12–50 folds higher than that of free doxorubicin [30].

12.3.1.3 HPMA Copolymer–Paclitaxel

Paclitaxel is one of the most effective anticancer agents known for the treatment of ovarian cancer, breast cancer, and lung cancer. For the effective delivery of the paclitaxel, HPMA copolymers are covalently conjugated which helps in solubilization of the drug. In the conjugation system paclitaxel was linked through tetrapeptide linker to the HPMA backbone copolymer chain. Meerum Terwogt et al. carried out the phase I study, where HPMA copolymer–paclitaxel conjugates illustrated antitumor activity in 12 patients having refractory breast cancer and skin metastases. The plasma pharmacokinetics was measured over 48 h which was linear with dose both for HPMA copolymer–paclitaxel conjugates and the native paclitaxel [31]. In another study, in vitro cytotoxicity study was carried using HPMA copolymer–gemcitabine–paclitaxel conjugate in human ovarian cancer cells (A2780) where the polymer–combined drug conjugate demonstrated synergistic effect, as compared to physical mixture of single polymer–drug conjugate [32].

12.3.1.4 HPMA Copolymer–Camptothecin

Camptothecin is an important class of potent antineoplastic drug having strong cytotoxicity against a wide range of tumors. The topoisomerase I enzyme was the cellular target of camptothecin and that the lactone function is essential for the antitumor activity [33, 34]. The lactone ring preferentially bind to the carboxylate present in serum albumin, camptothecin becomes unstable in the biological systems. This problem was overcome by conjugating camptothecin to HPMA copolymer, which helped in the improvement of the stability of the lactone ring. The C-20 hydroxyl group of camptothecin was attached to HPMA copolymer by ester linkage. The biological half-life of HPMA copolymer–camptothecin was increased up to more than 6 days, depicting its controlled release activity from conjugate system. To seek evidence of tumor targeting, a pilot clinical study was conducted involving ten patients with localized colorectal cancer. They were given HPMA copolymer–camptothecin before surgical removal of the primary tumor. After specified time intervals, the plasma, tumor, and adjacent normal tissue samples were analysed for free and polymer-bound drug. At 24 h after dosing no significant tumor targeting was observed. Further very negligible amount of free camptothecin levels were observed. However, these data's were not consistent with the clinical gamma camera imaging reported. Based on the phase I clinical results and the lack of evidence for tumor targeting, the clinical development of this polymer conjugate were abandoned.

12.3.1.5 Other HPMA Copolymer–Drug Conjugates

HPMA copolymer–aminoglutethimide–doxorubicin was used for metastatic breast cancer. Metastatic breast cancer is incurable but often responsive to treatment. Patients with estrogen-receptor-positive breast cancer may benefit more from endocrine-chemotherapy. Aminoglutethimide is a steroid derived from the cholesterol, which acts as anti steroid drug against metastatic breast cancer. The combination of the steroid with doxorubicin can be used as combinatorial endocrine-chemotherapy. With the use of the HPMA copolymer both the drugs were conjugated and can be simultaneously delivered. Vicent et al. and Greco et al. illustrated in vitro study of the HPMA copolymer–aminoglutethimide–doxorubicin conjugates illustrating enhanced cytotoxicity as compared to HPMA copolymer–doxorubicin in MCF-7 and aromatase-transfected MCF-7Ca cell lines [35–37]. Another polymer conjugate, HPMA copolymer–aminopropyl geldanamycin was used for an antibiotic having anticancer property. Geldanamycin belongs to benzoquinone ansamycin antibiotic having anticancer activity. It binds to heat shock proteins 90 [38] and GRP94 [39], inhibiting their capacity to form complexes with biological oncoproteins, such as p53 protein. The aminopropyl derivative of the geldanamycin, 17-allylamino-17-demethoxygeldanamycin was selected for phase I

clinical trial. Conjugating with HPMA copolymer, it showed modified mechanism of action and decreased nonspecific side effects as compared to the free geldanamycin. Kasuya et al. showed the conjugation of the OV-TL16 monoclonal antibody to HPMA- geldanamycin have more specific and targeted action on the A2780 and OVCAR-3 human ovarian carcinoma cells [40]. Similarly, Greish et al. and Borgman et al. synthesised the HPMA copolymer–aminohexyl geldanamycin conjugates along with antibody conjugation and illustrated more antiproliferative properties towards prostate cancer [41, 42]. Angiogenesis is crucial for tumor growth. Inhibitors of angiogenesis are used as anticancer drugs for the cancer therapy. Folkman and coworkers synthesized and developed polymer conjugate such as HPMA copolymer–O-(chloracetyl-carbamoyl) fumagillol to target and inhibit angiogenesis. This conjugate showed enhanced accumulation due to EPR effect in in vivo tumor and hepatectomy models [43].

12.3.2 Poly(α,L-glutamic acid)–Drug Conjugates

Poly(α,L-glutamic acid) (PG) composed of L-glutamic acid linked together through amide bonds. The pendent free γ-carboxyl group in each repeating unit of L-glutamic acid is negatively charged at neutral pH, which makes the polymer water soluble. PG was found to be more susceptible to lysosomal degradation than poly(L-aspartic acid) and poly(D-glutamic acid). When PG copolymers are sufficiently hydrophilic, they are eliminated primarily through the renal route with limited deposition in the cells of the reticuloendothelial system. Considering these advantageous factors, researchers have given attention towards the development of different PG–drug conjugates. The Poly(α,L-glutamic acid) is non immunogenic and nontoxic, hence Poly(α,L-glutamic acid)–drug conjugates are more deeply investigated in clinical trials.

12.3.2.1 Poly(L-glutamic acid)–Paclitaxel (PG-TXL)

To increase the water solubility and biodistribution, paclitaxel was conjugated with poly(L-glutamic acid), which demonstrated significantly reduced systemic toxicity and remarkable antitumor efficacy, including complete regression of well-established solid tumors in vivo. The antitumor and antimetastatic activities of PG-TXL were studied in four syngeneic murine tumors (breast MCa-4, breast MCa-35, hepatocarcinoma HCa-1, and sarcoma FSa-II) and all tumor models showed significantly better antitumor activities than that of free paclitaxel. The half-life of the PG-TXL was also prolonged ($t_{1/2}$ = 317 min) as compared to free paclitaxel ($t_{1/2}$ = 29 min). Todd et al. carried out the clinical study of PG-TXL, which was detectable in the plasma of all patients having a long plasma half-life of up to 185 h [44, 45]. Based on these results, PG-TXL was subsequently studied in phase I/II

trials. From the clinical findings, it was concluded that the PG-TXL can be delivered through parenteral route in lesser time than that of native paclitaxel (Taxol®) with good patient compliance.

12.3.2.2 Poly(L-glutamic acid)–Camptothecin

poly(L-glutamic acid) (PG) was conjugated at the C20(S) position of camptothecin through ester bonds. PG-camptothecin illustrated delayed growth in H322 human tumor xenograft models which showed fourfold increase in survival rate, as PG protects the lactone structure in camptothecin from the rapid ring-opening process [46]. Another group of researchers demonstrated that, by using the glycine linker between camptothecin and PG, the payload of the drug was increased up to 50 % by weight. There was an improved antitumor effect in B16 murine melanoma tumor model [47].

12.3.2.3 Poly(L-glutamic acid)–Anthracyclines

Anthracyclines (i.e., Doxorubicin, Daunorubicin) compounds are used to treat many cancers. The non-selective reactions with a variety of biomolecules, such as proteins and phospholipids inside the body during its course of action, limits their further clinical applications [48, 49]. Doxorubicin was conjugated to poly(L-glutamic acid) (PG) with an assumption that the conjugates can have greater selectivity and degrade to release Doxorubicin after they are endocytosed by tumor cells. Thus, Doxorubicin was conjugated to PG either directly or through oligopeptide spacers (Gly-Gly-Lec or Gly-Gly-Gly-Leu) via amide bonds. Anticancer activity increased with increasing oligopeptide length and degradation rate of the conjugate. The other potent drug, Daunorubicin inhibits the progression of the enzyme topoisomerase II by breaking the DNA chain replication and thereby stopping the process of cell replication. Daunorubicin have been attached to PG via hydrolytically labile ester bonds and hydrazone bonds. The hydrazone linkage formed by condensing the methylketone in Daunorubicin with hydrazide derivatized PG. It was found that the conjugate was active but less potent then free drug as determined by a [3H]uridine incorporation assay in Yac lymphoma bearing mice model [50].

12.3.2.4 Other Poly(L-glutamic acid) Polymer–Drug Conjugates

Poly (L-glutamic acid) (PG)–1-b-D-arabinofuranosylcytosine polymer conjugate for leukemia, therapy. 1-b-D-Arabinofuranosylcytosine (Ara-C) get conjugated with PG via amide bonds, where N-4 of Ara-C directly combined with the carboxyl

groups of PG and a conjugate in which Ara-C is linked via the aminoalkylphosphoryl side-chain introduced at C-5′ of Ara-C. Studies conducted in murine leukemia L1210 cells where the drug–polymer conjugate illustrated greater antitumor activity than that of the free Ara-C, due to the slow cleavage of free Ara-C from the conjugates and protection of Ara-C from deactivation by cytidine deaminase [51]. Poly(L-glutamic acid) (PG)–Mitomycin C conjugate was developed for leukemia therapy. Mitomycin isolated from *Streptomyces caespitosus* or *Streptomyces lavendulae*, which is also used as a chemotherapeutic agent by acting as a potent DNA cross linker. The Mitomycin C was also conjugated to PG through its aziridine amine using carbodiimide. The in vitro cytotoxicity, and in vivo studies of PG-Mitomycin C conjugates showed higher antitumor activity as compared to native Mitomycin C [52].

12.3.3 Poly(ethylene glycol)–Drug Conjugate

The poly (ethylene glycol) (PEG) was widely used commercial polymer approved by FDA. The specified molecular weight PEG has been developed in large scale. Further, the conjugation was possible by the different structural modifications to PEG backbone. The most important feature of PEG modification is that it greatly extends the half-life ($t_{1/2}$) of most proteins or drugs, and results in increased plasma presence. The PEG–protein conjugates, i.e., PEG–asparaginase (Oncaspar®) [53], PEG–adenosine deaminase (Adagen®) [54], PEG–interferon α-2a (Pegasys®) [55], PEG–interferon α-2b (PEG-Intron®) [56], and PEG growth hormone receptor antagonist (Somavert®) [57] have gained importance due to the reduced uptake by the reticuloendothelial system (RES). Like that the PEG-drug conjugates were exploited to modify the pharmacodynamic and pharmacokinetic properties of the anticancer drugs. The multiarm-PEG is used for the pegylation of the drugs, which ultimately increases the drug payload per PEG molecule as well as the biodegradation of the polymers [58, 59].

12.3.3.1 PEG-camptothecin

As described earlier, camptothecin acts as Topoisomerase I inhibitor in cancer chemotherapy. The PEG was conjugated with camptothecin by alaninate ester linkage at C-20-OH position, which favors the desired lactone ring configuration. Prothecan® (PEG40–Camptothecin conjugate) is now at phase II clinical trials. Enzon Pharmaceuticals, Inc. developed EZN-2208®, which has dendron like structure at the PEGs end chain and used to conjugate SN38 (active metabolite of camptothecin), now has entered to phase I clinical trial [60, 61]. The main limitation of PEG as drug carrier is the presence of only two reactive groups per polymer chain, which led to an intrinsically low drug payload. To overcome this limitation, the

construction of a dendron structure at the PEGs end chain has been proposed. Enzon is currently developing a conjugate of SN38, an active metabolite of camptothecin, with a 40-kDa PEG containing, which provide good results then the Prothecan®.

12.3.3.2 PEG-paclitaxel

PEG-paclitaxel, non-ionic paclitaxel prodrug is highly water soluble (>20 mg equiv. paclitaxel/ml) [62]. PEG-paclitaxel conjugate has difference in antitumor activity related to the changes of PEG molecular size [63]. According to the results of many researchers, the high molecular weight PEG prodrug conjugated with PEG (Mw = 20,000) produce an improved therapeutic effect. Li and coworkers suggested that, in MCA-4 mammary tumor-bearing mice, a single dose of PEG-paclitaxel (40 mg equiv. paclitaxel/kg body weight) significantly delayed tumor growth. In vivo results showed that PEG-paclitaxel inhibited the growth of B16 melanoma cells to an extent similar to that of paclitaxel, where the tumor growth rate was delayed by 0.9 days in case of PEG- paclitaxel conjugated animal as compared to free paclitaxel treated animals [64].

12.3.3.3 PEG–Gemcitabine

Gemcitabine is generally used in lung cancer, pancreatic cancer and breast cancer. However, it shows good results in patients with pancreatic cancer who have successful tumor resections. But the efficacy of the gemcitabine was lowered due to short half-life in blood circulation and rapid metabolism. The conjugation of gemcitabine to PEG demonstrated increased water solubility of the drug which in turn led to higher biodistribution and cytotoxicity. The recent studies on the PEG–gemcitabine conjugate showed a marked improvement in the cytotoxicity and apoptosis-inducing activity in MIA PaCa 2 and PANC 1 pancreatic cancer cell lines [65].

12.3.3.4 Other PEG Polymer–Drug Conjugates

Oridonin has potent antitumor activity, but it possesses rapid plasma clearance and high hydrophobicity. To make it substantially useful for chemotherapy it was conjugated with the PEG polymer. Shen et al. synthesized the PEG–oridonin conjugate by using the succinic acid as spacer moiety. The in vitro results illustrated satisfactory aqueous solubility which further increases with decreased molecular weight of PEG, while more significant sustained-release effect was shown with high molecular weight PEG. In vivo pharmacokinetic studies demonstrated that the elimination half-life was prolonged in comparison with oridonin solution [66]. The other anticancer drug prednisolone, belongs to glucocorticoid class, when inhaled for asthma, bronchitis treatments it is absorbed to blood and decreases its residence

time in lungs. The limitations can be overcome by ester conjugation of prednisolone to PEG. Bayard et al. synthesized PEG–prednisolone conjugate and showed that these conjugates are stable in buffers with a hydrolysis half-lives ranging from 1 to 70 h, depending on the pH and level of substitution. Whereas, the PEG2000 and mPEG2000 conjugates have reduced the maximum prednisolone concentration in the perfusate (C_{max}) by 3.0- and 2.2-fold, respectively. The blood retention time was increased to 40 min as compared to free drug, where it is 20 min. This study demonstrated that hydrolysable PEG drug ester conjugates can be a promising approach for optimising the pharmacokinetic profile of small drugs delivered by inhalation [67].

12.3.4 Other Polymer–Drug Conjugates

There are other polymers which are used in conjugation with various anticancer drugs to enhance its therapeutic activity. Cyclodextrin-based polymer (CDP) has been developed for the improved biodistribution towards tumor tissue. The components of CDP are β-cyclodextrin and polyethylene glycol. Camptothecin is covalently attached to CDP through a glycine linker, which preserves its active form and increases its water solubility. For example, IT-101 is a camptothecin–polymer conjugate. After i.v administration this cyclodextrin based polymer–camptothecin conjugate illustrated prolonged plasma half-life and enhanced distribution in tumor tissues when compared to camptothecin alone. The polymer conjugate also demonstrated 160 fold higher accumulation of active camptothecin released as compared to camptothecin alone [68, 69]. Using mucoadhesive polymers, polymer–drug conjugates are designed for its absorption and prolonged residence time. Soepenberg et al. [70] used DE-310, DX-8951 (exatecan mesylate, a camptothecin analogue) is linked to carboxymethyl dextran (Mw = 340 kDa) via glycyl-glycyl-phenylalanyl-glycyl-peptidyl spacer. The spacer used provides sustained release activity of the active moiety DX-8951 within the tumor as a result of enzymatic cleavage of the peptide by cathepsin B and cathepsin L. Ma et al. developed a multifunctional polymeric carrier for co-delivery of gene and drug. A new cyclodextrin derivative containing poly(L-lysine) dendrons was prepared by the click conjugation of per-6-azido-β-cyclodextrin with propargyl focal point poly(L-lysine) dendron. This conjugate formed a stable nanocomplex with plasmid DNA and exhibit high gene transfection efficiency. To this methotrexate drug was loaded efficiently with sustained release activity. The cyclodextrin derivative may be used directly for the combinatorial delivery of nucleic acid and lipophilic anticancer drugs without a complicated micellization process [71]. Han and Davis, developed a mucic acid polymer (MAP) conjugated camptothecin (MAP-CPT) and herceptin conjugated MAP-CPT nanoparticles and evaluated the efficacy in mice bearing BT-474 human breast tumors. The mice treated with non-targeted MAP-CPT nanoparticles showed significant tumor growth inhibition as compared to camptothecin alone. However,

mice receiving antibody conjugated MAP-CPT illustrated complete tumor regression demonstrating higher efficacy [72].

12.4 Conclusions and Future Prospects

These nanosized multicomponent polymer–drug conjugates has proved potentialities by reaching market and some of them are currently in clinical studies. Their application in cancer treatment is a promising field with growing opportunities to achieve effective medical treatments. However many challenges still exists, which could provide space for further improvement of this technology. Future generation of the polymer–drug conjugates will have to meet number of challenges, such as development of novel polymers, versatile conjugation chemistry which will allow site-specific attachment of targeting molecules and polymerization method as well as improved pharmacokinetic properties to allow accurate control of advance drug therapy. Thus, the polymer–drug conjugate approach is expected to show its greater therapeutic outcome in near future.

Acknowledgements The author A.K.M. thanks the CSIR, Government of India, for providing SRF fellowship. F.D. thanks DST, Government of India for providing women scientist grant and S.K.S. thanks DBT, Government of India for the Tata Innovative fellowship.

References

1. Jain KK (2003) Nanodiagnostics: application of nanotechnology in molecular diagnostics. Expert Rev Mol Diagn 3:153–161
2. Labhasetwar V (2005) Nanotechnology for drug and gene therapy: the importance of understanding molecular mechanisms of delivery. Curr Opin Biotechnol 16:674–680
3. Kubik T, Bogunia-Kubik K, Sugisaka M (2005) Nanotechnology on duty in medical applications. Curr Pharm Biotechnol 6:17–33
4. Sahoo SK, Parveen S, Panda JJ (2007) The present and future of nanotechnology in human health care. Nanomedicine 3:20–31
5. Krauze MT, Forsayeth J, Park JW, Bankiewicz KS (2006) Real-time imaging and quantification of brain delivery of liposomes. Pharm Res 23:2493–2504
6. Sutton D, Nasongkla N, Blanco E, Gao J (2007) Functionalized micellar systems for cancer targeted drug delivery. Pharm Res 24:1029–1046
7. Sahoo SK, Labhasetwar V (2003) Nanotech approaches to drug delivery and imaging. Drug Discov Today 8:1112–1120
8. Duncan R (2003) The dawning era of polymer therapeutics. Nat Rev Drug Discov 2:347–360
9. Vasey PA, Kaye SB, Morrison R, Twelves C, Wilson P, Duncan R, Thomson AH, Murray LS, Hilditch TE, Murray T, Burtles S, Fraier D, Frigerio E, Cassidy J (1999) Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents-drug-polymer conjugates. Cancer Research Campaign Phase I/II Committee. Clin Cancer Res 5:83–94
10. Ringsdorf H (1975) Structure and properties of pharmacologically active polymers. J Polym Sci Symp 51:135–153

11. Gillies ER, Goodwin AP, Frechet JM (2004) Acetals as pH-sensitive linkages for drug delivery. Bioconjug Chem 15:1254–1263
12. Torchilin VP (2005) Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4:145–160
13. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 65:271–284
14. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46:6387–6392
15. Maeda H (2001) The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzyme Regul 41:189–207
16. Duncan R (1992) Drug-polymer conjugates: potential for improved chemotherapy. Anticancer Drugs 3:175–210
17. Jain RK (1998) The next frontier of molecular medicine: delivery of therapeutics. Nat Med 4:655–657
18. Petrak K (2005) Essential properties of drug-targeting delivery systems. Drug Discov Today 10:1667–1673
19. Chipman SD, Oldham FB, Pezzoni G, Singer JW (2006) Biological and clinical characterization of paclitaxel poliglumex (PPX, CT-2103), a macromolecular polymer-drug conjugate. Int J Nanomedicine 1:375–383
20. Kopecek J, Kopeckova P (2010) HPMA copolymers: origins, early developments, present, and future. Adv Drug Deliv Rev 62:122–149
21. Pasut G, Veronese FM (2009) PEG conjugates in clinical development or use as anticancer agents: an overview. Adv Drug Deliv Rev 61:1177–1188
22. Takakura Y, Takagi A, Hashida M, Sezaki H (1987) Disposition and tumor localization of mitomycin C-dextran conjugates in mice. Pharm Res 4:293–300
23. Greish K, Fang J, Inutsuka T, Nagamitsu A, Maeda H (2003) Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting. Clin Pharmacokinet 42:1089–1105
24. Caliceti P, Monfardini C, Sartore L, Schiavon O, Baccichetti F, Carlassare F, Veronese FM (1993) Preparation and properties of monomethoxy poly(ethylene glycol) doxorubicin conjugates linked by an amino acid or a peptide as spacer. Farmaco 48:919–932
25. Ray A, Larson N, Pike DB, Gruner M, Naik S, Bauer H, Malugin A, Greish K, Ghandehari H (2011) Comparison of active and passive targeting of docetaxel for prostate cancer therapy by HPMA copolymer-RGDfK conjugates. Mol Pharm 8:1090–1099
26. Tang A, Kopeikova P, Kopeckeva J (2003) Binding and cytotoxicity of HPMA copolymer conjugates to lymphocytes mediated by receptor-binding epitopes. Pharm Res 20:360–367
27. Kopecek J, Kopeckova P, Minko T, Lu Z (2000) HPMA copolymer-anticancer drug conjugates: design, activity, and mechanism of action. Eur J Pharm Biopharm 50:61–81
28. Malugin A, Kopeckova P, Kopecek J (2004) HPMA copolymer-bound doxorubicin induces apoptosis in human ovarian carcinoma cells by a Fas-independent pathway. Mol Pharm 1:174–182
29. Seymour LW, Ferry DR, Kerr DJ, Rea D, Whitlock M, Poyner R, Boivin C, Hesslewood S, Twelves C, Blackie R, Schatzlein A, Jodrell D, Bissett D, Calvert H, Lind M, Robbins A, Burtles S, Duncan R, Cassidy J (2009) Phase II studies of polymer-doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer. Int J Oncol 34:1629–1636
30. Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S, Kerr DJ (2002) Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin. J Clin Oncol 20:1668–1676
31. Meerum Terwogt JM, ten Bokkel Huinink WW, Schellens JH, Schot M, Mandjes IA, Zurlo MG, Rocchetti M, Rosing H, Koopman FJ, Beijnen JH (2001) Phase I clinical and pharmacokinetic study of PNU166945, a novel water-soluble polymer-conjugated prodrug of paclitaxel. Anticancer Drugs 12:315–323

32. Larson N, Yang J, Ray A, Cheney DL, Ghandehari H, Kopecek J (2013) Biodegradable multi-block poly(N-2-hydroxypropyl)methacrylamide gemcitabine and paclitaxel conjugates for ovarian cancer cell combination treatment. Int J Pharm 454:435–443
33. Chabot GG (1997) Clinical pharmacokinetics of irinotecan. Clin Pharmacokinet 33:245–259
34. Rivory LP, Robert J (1995) Molecular, cellular, and clinical aspects of the pharmacology of 20(S)camptothecin and its derivatives. Pharmacol Ther 68:269–296
35. Vicent MJ, Greco F, Nicholson RI, Paul A, Griffiths PC, Duncan R (2005) Polymer therapeutics designed for a combination therapy of hormone-dependent cancer. Angew Chem Int Ed Engl 44:4061–4066
36. Greco F, Vicent MJ, Gee S, Jones AT, Gee J, Nicholson RI, Duncan R (2007) Investigating the mechanism of enhanced cytotoxicity of HPMA copolymer-Dox-AGM in breast cancer cells. J Control Release 117:28–39
37. Greco F, Vicent MJ, Penning NA, Nicholson RI, Duncan R (2005) HPMA copolymer-aminoglutethimide conjugates inhibit aromatase in MCF-7 cell lines. J Drug Target 13:459–470
38. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91: 8324–8328
39. Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer J, Neckers L (1996) p185erbB2 binds to GRP94 in vivo. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. J Biol Chem 271:4974–4977
40. Kasuya Y, Lu ZR, Kopeckova P, Minko T, Tabibi SE, Kopecek J (2001) Synthesis and characterization of HPMA copolymer-aminopropylgeldanamycin conjugates. J Control Release 74:203–211
41. Greish K, Ray A, Bauer H, Larson N, Malugin A, Pike D, Haider M, Ghandehari H (2011) Anticancer and antiangiogenic activity of HPMA copolymer-aminohexylgeldanamycin-RGDfK conjugates for prostate cancer therapy. J Control Release 151:263–270
42. Borgman MP, Ray A, Kolhatkar RB, Sausville EA, Burger AM, Ghandehari H (2009) Targetable HPMA copolymer-aminohexylgeldanamycin conjugates for prostate cancer therapy. Pharm Res 26:1407–1418
43. Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, Corfas G, Folkman J (2004) Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470. Nat Med 10:255–261
44. Todd R, Sludden J, Boddy AV, Griffin MJ, Robson L, Cassidy J, Bissett D, Main M, Brannan MD, Elliott S, Fishwick K, Verrill M, Calver H (2001) Phase I and pharmaco-logical study of CT-2103, a poly(L-glutamic acid)–paclitaxel conjugate. ASCO (abstract 439)
45. Sabbatini P, Aghajanian C, Hensley M, Pezzulli S, Oflaherty C, Soignet S, Lovegren M, Esch J, Funt S, Oflaherty O, Soignet S, Lovegren M, Esch J, Funt S, Odujinrin O, Warner M, Bolton MG, Spriggs D (2001) Early findings in a Phase I study of PG–paclitaxel (CT-2103) in recurrent ovarian or primary peritoneal cancer. Proceedings of the AACR-NCI-EORTC international conference on molecular targets and cancer therapeutics abstract 470. p. 96
46. Zou Y, Wu QP, Tansey W, Chow D, Hung MC, Charnsangavej C, Wallace S, Li C (2001) Effectiveness of water soluble poly(L-glutamic acid)-camptothecin conjugate against resistant human lung cancer xenografted in nude mice. Int J Oncol 18:331–336
47. Singer JW, De Vries P, Bhatt R, Tulinsky J, Klein P, Li C, Milas L, Lewis RA, Wallace S (2000) Conjugation of camptothecins to poly-(L-glutamic acid). Ann N Y Acad Sci 922:136–150
48. Parveen S, Sahoo SK (2008) Polymeric nanoparticles for cancer therapy. J Drug Target 16:108–123
49. Zunino F, Pratesi G, Micheloni A, Cavalletti E, Sala F, Tofanetti O (1989) Protective effect of reduced glutathione against cisplatin-induced renal and systemic toxicity and its influence on the therapeutic activity of the antitumor drug. Chem Biol Interact 70:89–101

50. Zunino F, Savi G, Giuliani F, Gambetta R, Supino R, Tinelli S, Pezzoni G (1984) Comparison of antitumor effects of daunorubicin covalently linked to poly-L-amino acid carriers. Eur J Cancer Clin Oncol 20:421–425
51. Kato Y, Saito M, Fukushima H, Takeda Y, Hara T (1984) Antitumor activity of 1-beta-D-arabinofuranosylcytosine conjugated with polyglutamic acid and its derivative. Cancer Res 44:25–30
52. Roos CF, Satoshi M, Yoshinobu T, Mitsuru H, Hitoshi S (1984) Physicochemical and antitumor characteristics of some polyamino acid prodrugs of mitomycin C. Int J Pharm 22:75–87
53. Graham LM (2003) PEGASPARAGINASE: are view of clinical studies. Adv Drug Deliv Rev 55:1293–1302
54. Levy Y, Hershfield MS, Fernandez-Mejia C, Polmar SH, Scudiery D, Berger M, Sorensen RU (1988) Adenosine deaminase deficiency with late onset of recurrent infections: response to treatment with polyethylene glycol-modified adenosine deaminase. J Pediatr 113:312–317
55. Bailon P, Palleroni A, Schaffer CA, Spence CL, Fung WJ, Porter JE, Ehrlich GK, Pan W, Xu ZX, Modi MW, Farid A, Berthold W, Graves M (2001) Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. Bioconjug Chem 12:195–202
56. Wang YS, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF (2002) Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. Adv Drug Deliv Rev 54:547–570
57. Trainer PJ, Drake WM, Katznelson L, Freda PU, Herman-Bonert V, van der Lely AJ, Dimaraki EV, Stewart PM, Friend KE, Vance ML, Besser GM, Scarlett JA, Thorner MO, Parkinson C, Klibanski A, Powell JS, Barkan AL, Sheppard MC, Malsonado M, Rose DR, Clemmons DR, Johannsson G, Bengtsson BA, Stavrou S, Kleinberg DL, Cook DM, Phillips LS, Bidlingmaier M, Strasburger CJ, Hackett S, Zib K, Bennett WF, Davis RJ (2000) Treatment of acromegaly with the growth hormone-receptor antagonist pegvisomant. N Engl J Med 342:1171–1177
58. Rowinsky EK, Rizzo J, Ochoa L, Takimoto CH, Forouzesh B, Schwartz G, Hammond LA, Patnaik A, Kwiatek J, Goetz A, Denis L, McGuire J, Tolcher AW (2003) A phase I and pharmacokinetic study of pegylated camptothecin as a 1-hour infusion every 3 weeks in patients with advanced solid malignancies. J Clin Oncol 21:148–157
59. Nojima Y, Suzuki Y, Yoshida K, Abe F, Shiga T, Takeuchi T, Sugiyama A, Shimizu H, Sato A (2009) Lactoferrin conjugated with 40-kDa branched poly(ethylene glycol) has an improved circulating half-life. Pharm Res 26:2125–2132
60. Posey JA 3rd, Saif MW, Carlisle R, Goetz A, Rizzo J, Stevenson S, Rudoltz MS, Kwiatek J, Simmons P, Rowinsky EK, Takimoto CH, Tolcher AW (2005) Phase 1 study of weekly polyethylene glycol-camptothecin in patients with advanced solid tumors and lymphomas. Clin Cancer Res 11:7866–7871
61. Greenwald RB, Pendri A, Conover C, Gilbert C, Yang R, Xia J (1996) Drug delivery systems. 2. Camptothecin 20-O-poly(ethylene glycol) ester transport forms. J Med Chem 39:1938–1940
62. Arpicco S, Stella B, Schiavon O, Milla P, Zonari D, Cattel L (2013) Preparation and characterization of novel poly(ethylene glycol) paclitaxel derivatives. Int J Pharm 454:653–659
63. Mathew AE, Mejillano MR, Nath JP, Himes RH, Stella VJ (1992) Synthesis and evaluation of some water-soluble prodrugs and derivatives of taxol with antitumor activity. J Med Chem 35:145–151
64. Li C, Yu D, Inoue T, Yang DJ, Milas L, Hunter NR, Kim EE, Wallace S (1996) Synthesis and evaluation of water-soluble polyethylene glycol-paclitaxel conjugate as a paclitaxel prodrug. Anticancer Drugs 7:642–648
65. Vandana M, Sahoo SK (2010) Long circulation and cytotoxicity of PEGylated gemcitabine and its potential for the treatment of pancreatic cancer. Biomaterials 31:9340–9356
66. Shen J, Zhang D, Zhao Z, Jia L, Zheng D, Liu G, Hao L, Zhang Q, Tian X, Li C, Guo H (2013) Synthesis, characterization, in vitro and in vivo evaluation of PEGylated oridonin conjugates. Int J Pharm 4456(1):80–86

67. Bayard FJ, Thielemans W, Pritchard DI, Paine SW, Young SS, Backman P, Ewing P, Bosquillon C (2013) Polyethylene glycol-drug ester conjugates for prolonged retention of small inhaled drugs in the lung. J Control Release 171:234–240
68. Schluep T, Cheng J, Khin KT, Davis ME (2006) Pharmacokinetics and biodistribution of the camptothecin-polymer conjugate IT-101 in rats and tumor-bearing mice. Cancer Chemother Pharmacol 57:654–662
69. Schluep T, Hwang J, Cheng J, Heidel JD, Bartlett DW, Hollister B, Davis ME (2006) Preclinical efficacy of the camptothecin-polymer conjugate IT-101 in multiple cancer models. Clin Cancer Res 12:1606–1614
70. Soepenberg O, de Jonge MJ, Sparreboom A, de Bruin P, Eskens FA, de Heus G, Wanders J, Cheverton P, Ducharme MP, Verweij J (2005) Phase I and pharmacokinetic study of DE-310 in patients with advanced solid tumors. Clin Cancer Res 11:703–711
71. Ma D, Zhang HB, Chen YY, Lin JT, Zhang LM (2013) New cyclodextrin derivative containing poly(L-lysine) dendrons for gene and drug co-delivery. J Colloid Interface Sci 405:305–311
72. Han H, Davis ME (2013) Single-antibody, targeted nanoparticle delivery of camptothecin. Mol Pharm 10:2558–2567

Part VI
Carrier-Based Approaches for Targeted Drug Delivery

Chapter 13
Functionalized Lipid Particulates in Targeted Drug Delivery

Mangal S. Nagarsenker, Ankitkumar S. Jain, and Sanket M. Shah

13.1 Introduction

Lipids have been of major interest and importance in field of drug delivery systems. Their application in formulation science has been diverse and promising in different ways. The number of reports published each year on lipid based formulations and lipid based formulations in market establish critical role of lipids in drug delivery. Of considerable importance is the application of lipids in design of particulate systems which are amenable to surface modifications for improved drug/gene delivery. In the past few years, reports on different lipid based particulate systems have increased tremendously with major focus on liposomes and solid lipid nanoparticles (SLNs) [1–7]. Other lipid based particulate systems that are evaluated include nanostructured lipid carriers (NLCs) [6, 8–11], emulsions [12–17], lipid–drug conjugates (LDCs) and recently reported lipid nano-particulates in the form of LeciPlex [18, 19], and polymer-lipid hybrid nanoparticles (PLN) [20–22]. One of the various reasons responsible for success of lipids in formulation of particulate systems is their bio-compatible and biodegradable nature [2, 19, 23]. Thus, lipid based particulate systems can suitably be employed for delivery through not only noninvasive routes, such as oral and topical, but also through parenteral routes which are very demanding with reference to delivery system design.

Lipid particulate systems, over the years, have undergone stupendous modifications. Initially lipid particulate system of micron size were reported which were followed by lipid particulates in nanosize range. The nanosize range of particles made them capable of targeting tumors better, primarily because of enhanced

Author (Ankitkumar S. Jain and Sanket M. Shah) contributed equally with all other contributors.

M.S. Nagarsenker (✉) • A.S. Jain • S.M. Shah
Department of Pharmaceutics, Bombay College of Pharmacy, Mumbai, India
e-mail: mangal.nagarsenker@gmail.com; mangal@bcp.edu.in

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_13

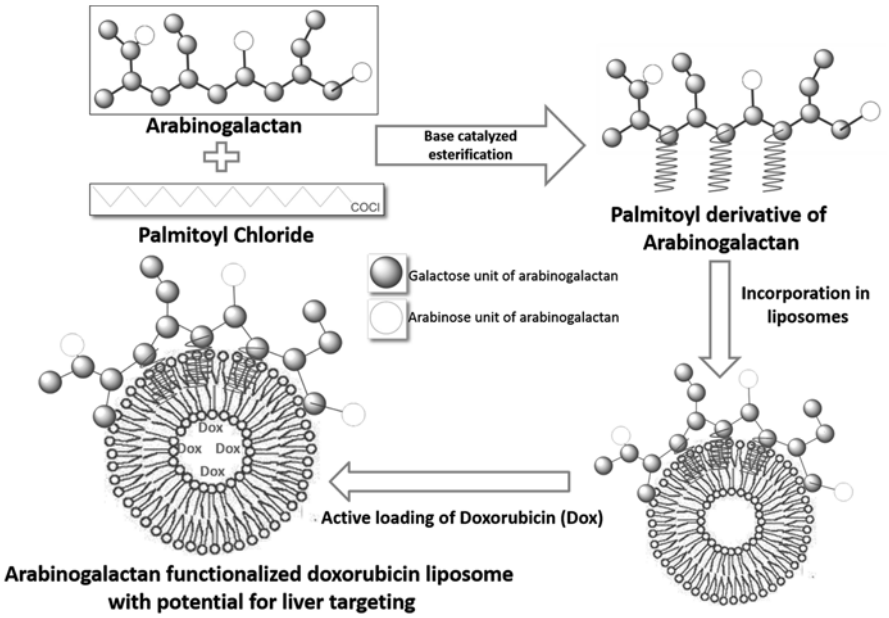

Fig. 13.1 Representation of one of the functionalized liposomal delivery systems [36]

permeation and retention (EPR) effect [24–31]. Subsequently the use of pH and/or temperature sensitive lipids to suit drug release in tumor environment made them more pronounced tool for tumor drug delivery [32, 33]. Another worthwhile modification was to render surface of lipid nanosystems more hydrophilic using different agents such as polyethylene glycols (PEGs) [33–35], which increased the systemic circulation time, resulting in prolonged drug release in plasma. Another significant achievement in lipid based particulate systems is conjugation of surface lipids to ligands that conferred ability to selectively target certain organs/tissue mass, and has been of great use in treatment of various infectious diseases and cancer in particular as depicted in Fig. 13.1 [3, 23, 25, 33, 36–45].

The chapter throws light on some key functional lipids employed in drug delivery and their final fate in human body. The chapter also focuses upon various lipid based particulate systems, their feasibility to functionalization and consequently their role in drug delivery.

13.2 Lipids as Excipients

The classification of lipids is very diverse. Lipids can be classified on the basis of their HLB values; their chemical structure such as glycerides, fatty acids, and so on; on the basis of their fatty acid chain length; depending on the charge they possess or even on the basis of their uses. The lipids discussed here are the ones predominantly employed in pharmaceutical formulations.

13.2.1 Phospholipid

Phospholipids comprise a huge family of lipids that have been instrumental in giving birth to a very important class of novel formulations known today, the vesicular drug delivery systems, better known as liposomes. Phospholipids are biocompatible as they are the key component of human cell membrane [46–51]. They are known to permeate skin as well as other cell membranes very effectively owing to structural similarity to cell membrane components [52–54]. Most phospholipids generally consist of a diglyceride, a phosphate group and a polar head group such as ethanolamine or choline, attached to phosphate moiety. This imparts them, both hydrophilic and hydrophobic regions in same molecule enabling them to act as efficient stabilizers at liquid–solid or liquid–immiscible liquid interface. Phospholipids have been extensively used as emulsifiers and stabilizers for a plethora of particulate systems such as nano-emulsions, micro-emulsions, polymeric and lipidic nanoparticles, and so on.

13.2.2 Liquid and Solid Lipids

Lipids comprise fatty acids, their monoglycerides, diglycerides, or triglycerides with C-chain length of 10 and more; waxes; sterols (cholesterol); and partial glycerides. Generally, short and medium chain glycerides fall in category of liquid lipids while long chain glycerides constitute most of solid lipids. Various solid lipids reported widely in recent literature reports include glyceryl behenate, glyceryl monostearate, glyceryl distearate, glyceryl dilaurate, glyceryl palmitostearate, cetyl palmitate, tristearin, tripalmitin, trimyristin, trilaurin, SOFTISAN® 142, long chain fatty acids like stearic acid, palmitic acid, decanoic acid, behenic acid, to name a few [1, 55–59]. Lipids help in better permeation of drug with poor permeability. Lipids also aid in improving solubilization of drug in gastrointestinal tract (GIT) by increase in micellization of drug by fatty acids liberated upon lipid digestion by pancreatic lipase and other enzymes [60–64]. Lipids also offer improvement in stability of photo- or pH dependent unstable drug [6, 19]. Importantly, both solid as well as liquid lipids, are amenable to chemical modifications imparting target specificity to them [23]. Some of these can be directly conjugated to active drug molecules to render a lipidic prodrug with better physicochemical properties which releases active molecule on reaching the desired site of action [65–67].

13.2.3 Lipids for Functionalization

Numerous reports are available on capability of functionalized lipids to improve drug delivery. Functionalization makes them either more target specific, or renders them a charge, or makes them capable of yielding pH/temperature-sensitive

nanoparticles or nanoparticles with greater plasma circulation half-life or a combination of all above mentioned attributes [68–74]. Attempts have been directed to conjugate hydrophilic molecules such as PEGs to lipids thus rendering stealth properties to the surface of nanoparticles fabricated from them. Conjugation of lipids to molecules possessing charge (+ve/−ve) renders them either a cationic or anionic charge, for instance dipalmitoyl phosphoglycerol (DPPG) imparts negative [75] and didodecyldimethylammonium bromide (DDAB) imparts positive charge [76] respectively. Various ligands based on immunoglobulins, carbohydrates, proteins, vitamins, and so on have been conjugated to lipids mentioned in greater detail later, to make them target specific. Another example of functionalizing lipids is of cholesteryl hemisuccinate molecule which is cholesterol ester of hemisuccinic acid which upon incorporation has been reported to impart pH sensitivity to the fabricated liposomes [77]. Functionalization of lipids has revolutionized the area of drug delivery, as elucidated, in the next sections of the chapter.

13.3 Fate of Lipid Particulate Systems

The major route of administration for a functionalized lipid based particulate system is the intravenous route, though a few functionalized lipid systems improve oral and transdermal absorption of actives. As is well known for metabolism of ingested lipids, and as is true for any lipid systems, whether functionalized or not, after oral delivery, lipids are acted upon by enzymes, especially pancreatic lipases, and the triglycerides are broken down to glycerol and free fatty acids or monoglycerides. After absorption in GIT, these free fatty acids are again reformed to triglycerides that are instrumental in formation of chylomicrons and lipoproteins. The lipoproteins are again responsible for utilization and excretion of cholesterol and also in formation of bile salts. The excess fatty acids is utilized for energy production inside the mitochondria of cells after their uptake as they enter TCA cycle.

Final fate of lipids even when administered intravenously as functionalized nanoparticles remains the same with a few differences. The functionalized lipid owing to target specificity reaches the target cells, helps in internalization of particles via receptor mediated endocytosis or carrier mediated uptake. However, once inside the cell, the biochemical pathway for the lipids remains the same as for lipids ingested by any other route, except that the targeted lipid is initially acted upon by enzymes to break the bond between the targeting ligand and lipid.

In addition to conferring targeting ability, lipid ligand employed for functionalization in lipid particulate system may influence in vivo course. It can render the surface of lipid nanosystems hydrophilic or charged depending on the property of ligand associated with lipid. This is beneficial with respect to reduced opsonization and RES uptake thus resulting in slower clearance and improved circulation times. PEGylation of lipids significantly improves circulation half-life of lipid nanosystems. The complex process of opsonization determines the eventual fate of the lipid

particulate system like its rate of clearance from the blood stream, volume distribution, organ distribution and its elimination from the body. PEGylation is an obligatory requirement to prolong circulation for delivery systems functionalized via antibodies, due to their known interaction with reticuloendothelial system leading to faster clearance of antibody functionalized delivery system from the circulation. Functionalization can change the pharmacokinetic and pharmacodynamics of the lipid based delivery system having a negative or positive effect on the therapeutic efficacy of the system [78].

13.4 Formulation Considerations for Functionalized Lipid Nanosystems

As in case of any formulation, the major concern for functionalized lipid nanosystem from point of view of formulation scientist is its ease to scalability and commercialization. Till the early 1990s, there were no high hopes about the viability of nanoparticulate based delivery systems in market, though the scenario started to change from late 1995, after introduction of Doxorubicin and Amphotericin B loaded liposomes (Doxil®, Caelyx®; and Ambisome®). Since then, various nanoparticulate systems, and predominantly lipid based nanosystems, have been introduced in market.

Today, functionalized lipid nanoparticles find themselves in similar situation as nanoparticles did two decades ago. But their future will depend on the ease of functionalization provided if those techniques can be scaled up without compromising the yield.

Tremendous efforts have been observed recently to effect functionalization of lipids to render delivery systems target specific. Ligands specific to receptors (targets) have been conjugated to lipids by either strong covalent bonds or weak van der Waal forces or hydrogen bonding. Covalent linkages generally employ esterification, amidation or sulfonation reactions between lipids and the ligand. Lipids used possess an acid, amine, sulfate, or alcohol functional group which is accordingly conjugated to its counterpart functional group/s on ligands. One important consideration during functionalization is that the ligand after being coupled to lipid should not show reduction in its affinity for target site. The alternative method of coupling ligands to fabricated lipid nanosystem as reported by many scientists is incubation or lyophilization of ligand together with the nanosystem. In such cases, the association of ligand to surface of lipid nanocarriers has been confirmed by various techniques, but predominantly by measurement of surface charge (zeta potential).

Literature also mentions use of linkers to couple ligand and lipid together. Linkers which are reported include pyridylditiopropionoylamino-PEG, hydrazide-PEG, maleimide-PEG, *p*-nitrophenylcarbonyl-PEG-PE, pyridylthiopropionoylamino-PEG-distearoylphosphatidylethanolamine (PDP-PEG-DSPE) to name a few [79, 80]. It is hypothesized that the linker allows the ligand to be extended from the

Table 13.1 Different ligands used for targeting

Category	Ligands	Receptor target	References
Immunoglobulins	Monoclonal antibodies	Overexpressed antigens on tumor cells	[79, 83–90]
	IgG	Overexpressed antigens on tumor cells	[90]
Carbohydrates	Sugars like galactose, mannose, fucose	Carbohydrate receptor	[91]
	Arabinogalactan	ASGPR (asialoglycoprotein receptor), a type of carbohydrate receptor	[36]
	Pullulan	ASGPR (asialoglycoprotein receptor), a type of carbohydrate receptor	[92, 93]
Proteins	Glycoproteins like transferrin, lectins	Transferrin receptor	[93, 94]
	RGD (arginine-glycine-aspartic acid oligopeptide)	αvβ3 integrins overexpressed mainly on endothelial cells	[95]
	Cell penetrating peptides such as TAT peptides	Interacts directly with lipid bilayer of cells to ensure faster cell internalization	[96]
Vitamins	folate or folic acid	Folate receptor	[37, 38, 70]
	Biotin	Biotin receptors overexpressed on tumor cells	[71]
Amino acids	Glutamic and aspartic acids	Amino acid receptors	[40]
	Arginine	Amino acid receptors	[97]
Actives/chemicals	Haloperidol, anisamide	Sigma receptors	[98, 99]
Positively charged lipids	Cetyltrimethyl ammonium bromide, Lipofectamine, didodecyldimethyl ammonium bromide	Negatively charged phospholipids overexpressed on tumor cells	[70]

surface for better interaction with receptors; however, ligand–lipid conjugates without linkers have also been observed to be equally effective in targeting [81].

Once the lipid–ligand conjugate is prepared, it is then incorporated in suitable amounts in the formula to ensure target specific lipid nanosystem is designed. Different percentages of conjugate are used in preparation of system and based on further in vitro experimentation; the right combination could be selected for further in vivo studies.

The various ligands which have been coupled to lipids as reported in literature are mentioned in Table 13.1 [25, 33, 82]

13.5 Route of Administration

The major concern with functionalized lipid particulates is toxicity. They should be safe at doses to be administered and for the route that they will be employed for. Most functionalized lipid particulates are designed to be administered by parenteral route (mainly intravenous delivery) in order to utilize their target specificity. Lipid particulates wherein surface is rendered more hydrophilic (by association with PEGs or gangliosides) or cationic (use of cationic lipids) are employed for intravenous delivery to improve plasma circulation time by minimizing opsonization and RES uptake. Positively charged lipid based delivery system are also known to improve oral absorption.

Functionalized lipid particulates have been employed for improving drug delivery through other routes including transdermal, pulmonary, vaginal, rectal, nasal, and ocular as reported in scientific literature (Table 13.2).

13.6 Applications in Drug Delivery

13.6.1 Liposomes

Liposomes are the most researched and preferred vesicular drug delivery system due to its versatility, safety, and in vivo advantage. Functionalization of liposomes is achieved via many techniques. The primary and the simplest technique is adsorption. In this technique, the preformed liposomal dispersion is incubated with the solution of targeting ligand. This approach of functionalization is not preferred as it is nonspecific and in most of the cases the rate of desorption is fast during storage as well as in vivo. Other technique of functionalization employs covalent link formation between targeting ligand and appropriate component of liposomes. This is usually achieved via chemical attachment of long carbon chain to functional groups like hydroxyl, carboxyl present on the targeting ligand. An important consideration

Table 13.2 Different routes of administration for functionalized lipid based nanoparticles

Type	Reported route/possible route of administration
Liposomes	
Stealth, galactosylated, mannosylated immunoliposomes, arabinogalactan associated liposomes, haloperidol anchored liposomes, folate conjugated liposomes	For improved intravenous and pulmonary delivery
Solid lipid nanoparticles	
Biotinylated	Improved delivery through oral and ocular epithelium
Galactosylated	Targeting via intravenous route
Nanostructured lipid carriers	
Squalene associated NLCs	Application to skin to achieve better percutaneous absorption
Transferrin associated NLCs	Targeting via intravenous delivery
Cholesterol rich NLCs	Intravenous delivery to target brain
Lipid drug conjugates	Oral/Parenteral route
Nano- and micro-emulsions	Oral/Intravenous route
Mucoadhesive nano-emulsions	Intranasal delivery for brain targeting
LeciPlex, Invasomes, Ethosomes, Transfersomes	Mainly evaluated for topical delivery
Polymer–lipid hybrid nanoparticles	For intravenous delivery
e.g., Lecithmer, Lipomer	

for chemical modification is that the groups chosen for chemical modification do not alter the receptor ligand interaction significantly. Another technique for functionalization involves chemical modification of preformed liposomes. In this technique, the preformed liposomes are incubated with linkers that covalently bind to the phospholipid head groups present on the surface of liposomes. These linker tagged liposomes after separation are treated with a solution of targeting ligand wherein the free end of the linker covalently attaches itself to the functional group present on targeting ligand, imparting targeting ability to liposomes.

Vodovozova et al. showed improvement in efficacy of a synthetic drug octadecylmerphalan after its incorporation in a liposomal delivery system functionalized by use of lectin specific carbohydrate ligand Sialyl Lewis X using its 3-aminopropyl glycoside derivative [65]. In vivo results confirmed superior therapeutic efficacy of Lectin functionalized liposomes as compared to liposomes devoid of it. Tsuruta et al. successfully loaded doxorubicin in an actively targeted liposomal delivery system using Sialyl Lewis X for preventing stenosis after angioplasty [100]. Study established superior activity of doxorubicin liposomes functionalized with Sialyl Lewis X as rats treated with functionalized liposomes had larger lumen area as compared to those treated with Doxorubicin liposomes. Kawakami et al. investigated the effect of glycosylation using galactose, mannose, and fucose on the clearance of liposomes. They concluded that galactose coated liposomes were taken up by the asialoglycoprotein receptor of the parenchymal cells of liver, mannose coated

liposomes were taken up by the mannose receptor on the non-parenchymal cells of the liver and fucose coated liposomes were taken up by the fucose receptor on the non-parenchymal cells of the liver. A higher molar concentration of galactose coated liposomes were also taken up by the non-parenchymal cells of the liver [91]. Shah et al. successfully fabricated actively targeted liposomal delivery system using asialoglycoprotein receptor specific arabinogalactan as the targeting ligand using covalent link to lipid component [36]. For further reading on use of carbohydrate mediated liposomal targeting, readers are referred to an excellent review by Malcolm N. Jones [101].

Wolff and Gregoriadis successfully fabricated monoclonal anti-Thy1IgG1 coated immunoliposomes for targeting to AKR-A cells [90]. Debs et al. successfully fabricated anti-Thy 1.1 monoclonal antibody MRCOX7 conjugated liposomes which demonstrated enhanced uptake in lymph nodes that express high levels of target antigen [84]. Koning et al. showed high intracellular delivery of cytotoxic agent by developing immunoliposomes using monoclonal antibody against rat colon carcinoma containing 5-fluorodeoxyuridine as the cytotoxic agent [86]. Suzuki et al. developed long circulating immunoliposome containing doxorubicin using murine monoclonal antibody HBJ127 that recognizes a peptide epitope of gp125 which is expressed on almost all human cancer cells [88, 89]. Mercadal et al. fabricated My-10 monoclonal antibody coated immunoliposome against CD34 antigen using carboxyfluorescein as marker compound [102]. Yang et al. formulated PEGylated immunoliposome loaded with paclitaxel using Herceptin as targeting ligand for cells overexpressing human epidermal growth factor receptor 2 [103]. Lukyanov et al. modified commercially available doxorubicin loaded long circulating liposomes Doxil® with monoclonal nucleosome specific 2C5 antibody that identifies tumors via surface bound nucleosomes [85, 87]. Biswas et al. reported enhanced suppression of tumor in vivo by surface functionalization of doxorubicin loaded long circulating liposome Doxil® using a cell penetrating peptide Octa-arginine [97].

Kitagawa and Kasamaki improved the intradermal delivery of retinoic acid using positively charged liposomes functionalized with 1,2-dioleoyl-3-trimethylammonium propane as cationic surfactant [104]. Knudsen et al. demonstrated improved delivery of calcipotriol by formulating and altering the fluid state of liposomes composed of dipalmitoyl phosphocholine and dilauroyl phosphocholine [105]. Geusens et al. developed ultradeformable cationic liposomes composed of cationic lipid 1,2-dioleoyl-3-trimethylammonium propane and the edge activator sodium cholate for delivery if siRNA into human primary melanocytes [106].

13.6.2 Solid Lipid Nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs)

SLNs and NLCs have been reported to a good extent for functionalization and targeting. Modifications are reported either by functionalizing the lipid that is employed for SLN/NLC preparation or the surfactant that is employed in its colloidal

stabilization. Xu et al. designed hepatoma targeted SLN for effective delivery of docetaxel wherein galactosylated-DOPE was utilized for site-specific delivery [23]. The authors report the potential of galactosylated SLNs in effective treatment of locally advanced as well as metastatic hepatocellular carcinoma. In comparison to non galactosylated SLNs, galactosylated-SLNs demonstrated no significant difference in mean particle size, zeta potential, drug loading, and entrapment efficiency. However, remarkable difference was noted in in vitro cytotoxicity and biodistribution of docetaxel when loaded in targeted SLN as compared to normal SLN. Galactosylated SLNs were superior to nongalactosylated SLN as well as marketed Taxotere formulation for improved delivery of Docetaxel.

Alukda et al. fabricated functionalized SLNs with coats of PLL (polylysine) and heparin, as a delivery template, loaded with vaginal microbicide (tenofovir) for prevention of HIV transmission [39]. The authors reported improved therapeutic efficacy of functionalized SLN as compared to nonfunctionalized SLN with no cytotoxicity to vaginal epithelium.

Yet another interesting example is of Kashanian et al. who functionalized SLN with *N*-glutaryl phosphatidylethanolamine and employed in the design of SLN to render the SLN pH sensitive, for enhancement in drug release in acidic pH of tumor [40]. When studied under different pH conditions, pH sensitive SLNs exhibited higher in vitro drug release at acidic pH.

NLCs were designed as a modification of SLN to improve their drug loading capacity, improve their colloidal stability, and decrease drug leakage during shelf life of product. They comprise both liquid and solid lipids unlike their SLN counterparts that make them comparatively more versatile as a drug delivery vehicle. NLCs have also been reported to be amenable to functionalization. Yang et al. prepared hyaluronic acid coated NLCs for targeted delivery of Paclitaxel to CD44 overexpressed on tumor cells. The study reports that functionalized NLCs were superior to Taxol in vitro as well as in vivo. Earlier Chen et al. had also published their work for targeted delivery of Paclitaxel. They conjugated Stearyl-2-amino-2-deoxyglucose (2-DG), a glucosamine derivative serving as a broad tumor targeting ligand, to glyceryl monostearate and oleic acid NLCs, for improved delivery of Paclitaxel to tumor cells.

Folate and transferrin have also been anchored to lipid nanoparticles for establishing tumor specific delivery of etoposide [94]. As mentioned earlier, choice of excipients can also render delivery system like SLN/NLCs target specific. Goppert and Muller discovered that SLN stabilized by Tween 80 adsorbs such plasma proteins as may be required for efficient targeting to brain, whereas Poloxamer 188 stabilized SLN adsorb proteins which imparted ability to prolong plasma circulation time [107]. Several reports have been published about binding of Tween 80 to apolipoproteins in plasma and thus capable of targeting and traversing blood–brain barrier. Incorporation of such functional surfactants confers target specificity to delivery system. Similarly, Rezazadeh and coworkers designed tumor targeted NLCs composed of cholesterol, known for targeting LDL receptors overexpressed on tumor cells [108, 109].

13.6.3 Nano- and Micro-emulsions

Not many reports are available with respect to functionalization of emulsions involving chemical reactions. However, use of specific surfactants possessing charge, negative or positive, in fabrication of charged nanoglobules of emulsions has been examined for improving bioavailability of actives as well as in gene delivery.

13.6.4 Miscellaneous Functionalized Lipid Based Systems

PLNs are a class of lipid nanocarriers that have surfaced up about a decade ago and have demonstrated capabilities to functionalization. PLN was viewed as an attempt to combine advantages and avoid disadvantages of polymeric nanoparticles and liposomes. Though dependent on method of preparation, general PLN description mentions presence of a polymer core coated with lecithin (or other lipid) which in turn can be conjugated/anchored to a ligand. Liu Y. et al. demonstrated that PLN composed of PLGA as polymer and mix of lipids, including PEGylated as well as folate conjugated PEGylated phospholipid [110]. The folate and PEG functionalized PLN when loaded with Docetaxel released ~18 % which was at surface of targeted PLN within first 12 h as would be required to exert immediate action on cancer cells. Thereafter, however, release was considerably slow, with only ~60 % Docetaxel released at the end of 72 h and sustained release continued for 168 h. The in vitro release study results correlated well with results of in vitro cytotoxicity study as well as cell uptake study, demonstrating ability of functionalized PLN to improve drug uptake possibly due to receptor mediated endocytosis.

Selection of polymer also in its own way contributes to functionalization of PLNs. Wu and coworkers demonstrated that use of soybean oil based anionic polymer provided adequate loading of cationic drugs such as Doxorubicin HCl due to ionic complexation in PLN in comparison to their SLN counterparts which lack in polymer content [22].

However, one of the interesting studies on PLN was reported by Clawson et al. who reported fabrication of PLN with pH triggered erosion of PEG shell covering PLN coat. The group designed PLN system for effective treatment of cancer with ability to erode the coat of PEG in response to low pH at tumor site. The targeting lipid (succinate ester based PEGylated lipid) used in design of PLN was synthesized by reacting 1,2-dipalmitoyl-*sn*-glycero-3-phospho(ethylene glycol) with methoxy polyethylene glycol. At neutral pH (physiological pH), the PEG surface coat will be more stable to hydrolysis whereas, in acidic tumor environment, due to erosion of PEG coat, the system will destabilize and show better fusion with cells at tumor site. However, it was worth noting that more the amount of synthesized functional lipid

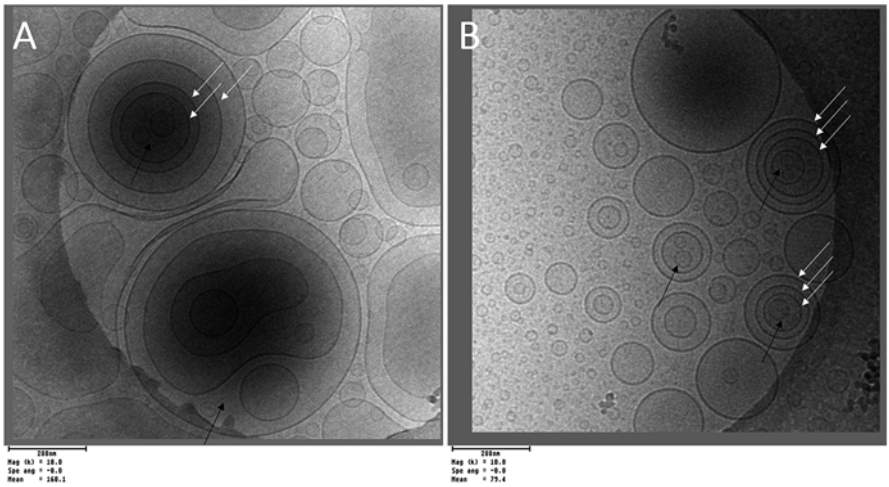

Fig. 13.2 CryoTEM images of LeciPlex system comprising cetyl trimethyl ammonium bromide (**a**) and didodecyl dimethyl ammonium bromide (**b**) as charged lipids. These are multivesicular (*black arrow*) multilamellar (*white arrow*) vesicles having a unique structure. They are unlike the classical multilamellar vesicles as each lamella is well differentiated giving an appearance of concentric rings

used in fabricating the PLN system, more stable was the developed PLN system to aggregation and destabilization, possibly due to greater number of ester bonds which need to be hydrolysed. Thus, PLN with 15 mol% functional lipid were not stable at pH below 6.0, with 20 mol %, stability was lost at pH 5.0, with 30 mol %, particles retained stability until pH dropped to 4.0, with 40 mol %, PLN do not destabilize until pH drops to 3.0 and with 50 mol %, PLN system was stable over entire pH ranges evaluated (pH 7.4–3.0).

A great emphasis has been placed on lipid based nanosystems which are amenable to surface functionalization that aids in rendering them target/site specific. However, several novel lipid based nanosystems can be considered functionally distinct from the others owing to their potential in improving delivery of actives. These include various deformable/flexible vesicular systems like LeciPlex, transfersomes, invasomes, and ethosomes. Though all of these are vesicular in nature, the systems do possess notable differences imparting them certain specific characteristics.

LeciPlex is a novel vesicular system that combines advantages of cationic nanoparticles with those of vesicular systems (Fig. 13.2). The distinct advantage of LeciPlex system is ease of formulation and its amenability to scale-up. It employs a single step fabrication involving addition of aqueous phase to solution of phospholipid and cationic agent in a biocompatible solvent leading to formation of vesicular system under simple agitation [18, 19]. LeciPlex are cationic vesicles, and

therefore capable of loading both hydrophilic as well as lipophilic drugs, and their cationic nature confers better colloidal stability to the system. Besides, the cationic charge allows them capability to target cancer cells overexpressing negatively charged molecules at their surface [111]. Also, the agents that impart positive charge to the vesicles possess selective cytotoxicity to various cancer cell lines. This makes LeciPlex a useful addition to drug delivery systems for cancer treatment. LeciPlex has been implicated in improving oral bioavailability of actives with different polarities, such as Repaglinide and Quercetin. We have established the utility of LeciPlex in condensing DNA for gene delivery. Besides oral delivery, LeciPlex holds a lot of potential for effective topical delivery, the experimentation for which are under way.

Deformable liposomes, ultradeformable liposomes, ethosomes, and invasomes are all, another important sect of functionalized vesicular carriers that have been mainly explored for dermal/transdermal delivery. Transferosomes consist of flexible bilayers and are the first generation of elastic liposomes [112, 113]. These are reported to enhance skin permeation of loaded actives through intact skin under influence of transdermal hydration and osmotic gradients, when applied under non-occluded conditions [113, 114]. The innovative addition in engineering Transferosomes as a different vesicular carrier than conventional liposomes has been addition of an edge activator. The edge activator is a molecule that provides desired flexibility and deformability to the lipid bilayers to improve its skin permeation wherein the vesicle is able to move into skin layers in intact form, thus improving flux of loaded actives [115]. Examples of edge activators include molecules such as sodium cholate, Span 80, Tween 80, and dipotassium glycyrrhizinate [116–118]. The second generation of deformable liposomes, named as proultraflexible liposomes, was reported by Jain et al., following the proliposomal approach known to enhance stability of vesicles. The formulation demonstrated better permeation of loaded active, levonorgestrel and better stability than proliposomal formulation [119]. Numerous reports are available that enlighten ability of deformable liposomes or their likes, such as ultradeformable liposomes or cationic ultradeformable liposome employing a cationic lipid [120] to improve not only skin permeation of actives, such as diclofenac, bleomycin [121], diclofenac [112], 5-FU [122], but also delivery of vaccines and genetic material like siRNA [106, 123].

Ethosomes, comprising phospholipids, a high ethanol content and water, are another specialized vesicular systems that are able to permeate into deeper layers of skin, as has been reported by few confocal laser scanning microscopy studies, due to their high malleability have improved systemic delivery of few actives. Ethosomes have reportedly improved skin permeation of various loaded agents including ketotifen, 5-aminolevulinic acid, and rhodamine red, to name a few [113, 124–129].

Yet another interesting vesicular carrier class comprises invasomes reported by Fahr A. et al. which in addition to phospholipids consists of mixture of terpenes that

act as permeation enhancers. The group reported fabrication of phospholipid based invasomes comprising 3.3 % ethanol and 1 % mixture of terpenes with size less than 150 nm, significantly improved Temoporfin deposition in stratum corneum as compared to conventional liposomes [130].

13.7 Marketed/Potential Lipid Particulates

There are many lipid particulates which have either entered clinical trials or have been successfully launched commercially [131]. In case of liposomal products, currently, there are 53 under therapeutic investigation and 8 liposomal products available commercially, most in comparison to any other lipid based system. There are 19 and 9 emulsion based products being available for therapeutic investigation and commercial use, respectively [132]. Table 13.3 mentions list of few of such functionalized lipid nanoparticulates which are successful or have promise to reach the market.

13.8 Summary and Conclusion

Functionalization of lipid particulates make them more promising for their intended use, such as enhanced permeation, increased target specificity, improved lipophilicity, and so on. A number of reports have been available recently on importance of functionalization of lipid nanosystems, as described earlier in the chapter. Task of functionalizing particulates renders excellent opportunity for formulation scientists and chemists to work together and lay foundation of successful novel drug delivery systems. Functionalized lipid nanoparticulates are expected to face the same challenge that was faced by lipid nanoparticulates a few years back with respect to their commercial feasibility. The efforts of multiple formulation scientists made the lipid based nanosystems a commercial success and it can be expected that functionalized lipid nanoparticles also reach market in increasing number and probability in near future.

Acknowledgments Ankitkumar S. Jain is thankful to Amrut Mody Research Fund (AMRF), Mumbai, India and University Grants Commission (UGC), New Delhi, India for financial assistance. Sanket M. Shah is thankful to Indian Council of Medical Research (ICMR), New Delhi, India for financial assistance. We would also like to extend our acknowledgement to Mukul Ashtikar, Frank Steiniger, Jana Thamm, and Prof. Alfred Fahr from Friedrich Schiller University, Jena, Germany for expertise and support in investigating the vesicular structure of LeciPlex, as a part of Indian Council of Medical Research ICMR–Bundesministerium für Bildung und Forschung BMBF funded project.

Table 13.3 Formulations under investigation/approved for functionalized lipid based nanoparticles

Name of product	Type of lipid particulate	Company/alliance	Status	Approved indications by FDA
Doxil (USA)	PEGylated doxorubicin HCl liposomes	Ortho Biotech	Available commercially	Metastatic ovarian cancer, AIDS related Kaposi's sarcoma
Caelyx (Outside USA)		Schering-Plough		
ThermoDox	Lyso thermo-sensitive liposomal doxorubicin	Celsion	Phase 2 and 3 clinical trial	Metastatic colorectal cancer and hepatocellular carcinoma
Lipoplatin™	Stealth liposome composed of SoyPC/DPPG/CHOL loaded with cisplatin	Regulon Inc.	Phase 3 clinical trial	Pancreatic cancer, lung cancer
EndoTAG-1	Positively charged liposomal paclitaxel	Medigene and SynCore Biotechnology	Phase 2 clinical trial	Pancreatic cancer and triple negative breast cancer
MBP-426	Oxaliplatin-encapsulated transferrin (Tf)-conjugated *N*-glutaryl phosphatidyl-ethanolamine (NGPE)-liposome	Mebiopharm Co., Ltd	Phase 2 clinical trial	Advanced or metastatic solid tumors, gastric adenocarcinoma, gastroesophageal adenocarcinoma, esophageal adenocarcinoma
SPI-077	Stealth liposome composed of SoyHPC/CHOL/DSPE-PEG loaded with Cisplatin	Sequus Pharmaceuticals	Phase 1/2 clinical trial	Head and neck cancer, lung cancer
C225-ILS-DOX	Anti-EGFR-immunoliposomes loaded with doxorubicin	University Hospital, Basel, Switzerland	Phase 1 clinical trial	Advanced solid tumors
MCC-465	Immunoliposome-encapsulated doxorubicin tagged with polyethylene glycol (PEG) and the F(ab′)2 fragment of human monoclonal antibody GAH	National Cancer Center, Tokyo	Phase 1 clinical trial	Stomach cancer
SGT53-01	Anti-transferrin receptor scFv tagged liposomal p53 DNA and docetaxel	SynerGene Therapeutics, Inc.	Phase 1 clinical trial	Treat solid tumors
S-CKD602	Stealth liposome containing camptothecin analog	Alza Co.	Phase 1	Several cancer types

References

1. Mehnert W, Mader K (2001) Solid lipid nanoparticles: production, characterization and applications. Adv Drug Del Rev 64(Suppl):83–101
2. Muller RH, Mader K, Gohla S (2000) Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur J Pharm Biopharm 50(1):161–177
3. Tranum, K. and S. Roderick, *Solid Lipid Nanoparticles: Tuneable Anti-Cancer Gene/Drug Delivery Systems*, in *Novel Gene Therapy Approaches*. 2013, INTECH. p. 53–73
4. Wong HL et al (2007) Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles. Adv Drug Deliv Rev 59(6):491–504
5. Pandey R, Sharma S, Khuller GK (2005) Oral solid lipid nanoparticle-based antitubercular chemotherapy. Tuberculosis 85(5–6):415–420
6. Jain AS et al (2013) Lipid colloidal carriers for improvement of anticancer activity of orally delivered quercetin: formulation, characterization and establishing in vitro-in vivo advantage. J Biomed Nanotechnol 9:1230–1240
7. Pathak P, Nagarsenker M (2009) Formulation and evaluation of lidocaine lipid nanosystems for dermal delivery. AAPS Pharm Sci Tech 10(3):985–992
8. Joshi MD, Müller RH (2009) Lipid nanoparticles for parenteral delivery of actives. Eur J Pharm Biopharm 71(2):161–172
9. Muller RH et al (2007) Nanostructured lipid carriers (NLC) in cosmetic dermal products. Adv Drug Deliv Rev 59(6):522–530
10. Pardeike J, Hommoss A, Muller RH (2009) Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. Int J Pharm 366(1–2):170–184
11. Teeranachaideekul V et al (2007) Cetyl palmitate-based NLC for topical delivery of Coenzyme Q10—development, physicochemical characterization and in vitro release studies. Eur J Pharm Biopharm 67(1):141–148
12. Date AA, Nagarsenker MS (2008) Parenteral microemulsions: an overview. Int J Pharm 355(1–2):19–30
13. Nagarsenker M, Tayade N (2010) Development and evaluation of artemether parenteral microemulsion. Indian J Pharm Sci 72:637–640
14. Dixit RP, Nagarsenker MS (2008) Formulation and in vivo evaluation of self-nanoemulsifying granules for oral delivery of a combination of ezetimibe and simvastatin. Drug Dev Ind Pharm 34(12):1285–1296
15. Date AA et al (2010) Self-nanoemulsifying drug delivery systems: formulation insights, applications and advances. Nanomedicine (Lond) 5(10):1595–1616
16. Dixit RP, Nagarsenker MS (2010) Optimized microemulsions and solid microemulsion systems of simvastatin: characterization and in vivo evaluation. J Pharm Sci 99(12):4892–4902
17. Ankitkumar S Jain et al (2011) Nanoemulsions: a potential delivery system. Pharma Rev.:113–119
18. Date AA et al (2011) Lecithin-based novel cationic nanocarriers (LeciPlex) I: fabrication, characterization and evaluation. Nanomedicine (Lond) 6(8):1309–1325
19. Date AA et al (2013) Lecithin-based novel cationic nanocarriers (leciplex) II: improving therapeutic efficacy of quercetin on oral administration. Mol Pharm 8(3):716–726
20. Benival DM, Devarajan PV (2011) Lipomer of doxorubicin hydrochloride for enhanced oral bioavailability. Int J Pharm 423(2):554–561
21. Dhumal R et al (2011) Evaluation of safety of lipomer doxycycline hydrochloride (lipomer DH). J Biomed Nanotechnol 7(1):146–147
22. Wong HL et al (2007) In vivo evaluation of a new polymer-lipid hybrid nanoparticle (PLN) formulation of doxorubicin in a murine solid tumor model. Eur J Pharm Biopharm 65(3):300–308
23. Xu Z et al (2009) The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma. Biomaterials 30(2):226–232

24. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46(12 (Part 1)):6387–6392
25. Danhier F, Feron O, Preat V (2010) To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. J Control Release 148(2):135–146
26. Roerdink FH, Dijkstra J, Spanjer HH, Scherphof GL (1984) Interaction of liposomes with hepatocytes and Kupffer cells in vivo and in vitro. Biochem Soc Trans 12:335–336
27. Palmer TN et al (1984) The mechanism of liposome accumulation in infarction. Biochim Biophys Acta 797(3):363–368
28. Jain RK (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. J Natl Cancer Inst 81(8):570–576
29. Torchilin VP (2000) Drug targeting. Eur J Pharm Sci 11(Suppl 2):S81–S91
30. Maeda H, Sawa T, Konno T (2001) Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. J Control Release 74(1–3):47–61
31. Maeda H, Bharate GY, Daruwalla J (2009) Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur J Pharm Biopharm 71(3):409–419
32. Ishida T et al (2001) Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells. Biochim Biophys Acta 1515(2):144–158
33. Marcucci F, Lefoulon FO (2004) Active targeting with particulate drug carriers in tumor therapy: fundamentals and recent progress. Drug Discov Today 9(5):219–228
34. Sant V, Nagarsenker M (2011) Synthesis of monomethoxypolyethyleneglycol-cholesteryl ester and effect of its incorporation in liposomes. AAPS Pharm Sci Tech 12(4):1056–1063
35. Papahadjopoulos D et al (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci U S A 88(24):11460–11464
36. Shah SM et al (2012) Synthesis, characterization, and in vitro evaluation of palmitoylated arabinogalactan with potential for liver targeting. Carbohydr Res 367:41–47
37. Saul JM et al (2003) Controlled targeting of liposomal doxorubicin via the folate receptor in vitro. J Control Release 92(1–2):49–67
38. Turk MJ, Waters DJ, Low PS (2004) Folate-conjugated liposomes preferentially target macrophages associated with ovarian carcinoma. Cancer Lett 213(2):165–172
39. Alukda D, Sturgis T, Youan B-BC (2011) Formulation of tenofovir-loaded functionalized solid lipid nanoparticles intended for HIV prevention. J Pharm Sci 100(8):3345–3356
40. Kashanian S, Azandaryani AH, Derakhshandeh K (2011) New surface-modified solid lipid nanoparticles using N-glutaryl phosphatidylethanolamine as the outer shell. Int J Nanomedicine 6:2393–2401
41. Maruyama K et al (1999) Possibility of active targeting to tumor tissues with liposomes. Adv Drug Deliv Rev 40(1–2):89–102
42. Wu J, Nantz MH, Zern MA (2002) Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications. Front Biosci 7:d717–d725
43. Mauk MR, Gamble RC, Gamble JD (1980) Targeting of lipid vesicles: specificity of carbohydrate receptor analogues for leukocytes in mice. Proc Natl Acad Sci U S A 77(8):4430–4434
44. Vyas SP et al (2000) Ligand directed macrophage targeting of amphotericin B loaded liposomes. Int J Pharm 210(1–2):1–14
45. Nobs L et al (2004) Current methods for attaching targeting ligands to liposomes and nanoparticles. J Pharm Sci 93(8):1980–1992
46. Choi J et al (2008) Surface immobilization of biocompatible phospholipid polymer multilayered hydrogel on titanium alloy. Colloids Surf B Biointerfaces 67(2):216–223
47. Lim SB, Rubinstein I, Onyuksel H (2008) Freeze drying of peptide drugs self-associated with long-circulating, biocompatible and biodegradable sterically stabilized phospholipid nanomicelles. Int J Pharm 356(1–2):345–350

48. Moro T et al (2008) 553 biocompatible phospholipid polymer grafting on liner surface of artificial hip joints enhances the wear resistance independently of liner cross-linking or femoral head material. Osteoarthr Cartilage 16(Suppl 4):S234
49. Moro T et al (2010) Surface grafting of biocompatible phospholipid polymer MPC provides wear resistance of tibial polyethylene insert in artificial knee joints. Osteoarthr Cartilage 18(9):1174–1182
50. Nguyen TTL et al (2010) Biocompatible lecithin-based microemulsions with rhamnolipid and sophorolipid biosurfactants: formulation and potential applications. J Colloid Interface Sci 348(2):498–504
51. Rubinstein I, Onyuksel H (2007) Biocompatible, biodegradable and sterically stabilized phospholipid nanomicelles improve cryopreservation of oral keratinocytes: a preliminary investigation. Int J Pharm 338(1–2):333–335
52. Azuma K et al (2002) Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. J Agric Food Chem 50(6):1706–1712
53. Chen C-C et al (2010) Effects of lipophilic emulsifiers on the oral administration of lovastatin from nanostructured lipid carriers: physicochemical characterization and pharmacokinetics. Eur J Pharm Biopharm 74(3):474–482
54. Gershanik T, Benzeno S, Benita S (1998) Interaction of a self-emulsifying lipid drug delivery system with the everted rat intestinal mucosa as a function of droplet size and surface charge. Pharm Res 15(6):863–869
55. Freitas C, Muller RH (1998) Spray-drying of solid lipid nanoparticles (SLNTM). Eur J Pharm Biopharm 46(2):145–151
56. Domb AJ (1995) Long acting injectable oxytetracycline-liposphere formulations. Int J Pharm 124(2):271–278
57. Westesen K, Bunjes H (1995) Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix? Int J Pharm 115(1):129–131
58. Almeida AJ, Runge S, Muller RH (1997) Peptide-loaded solid lipid nanoparticles (SLN): influence of production parameters. Int J Pharm 149(2):255–265
59. Gasco MR, Cavalli CME (1992) Timolol in lipospheres. Pharmazie 47(2):119–121
60. Dahan A, Hoffman A (2006) Use of a dynamic in vitro lipolysis model to rationalize oral formulation development for poor water soluble drugs: correlation with in vivo data and the relationship to intra-enterocyte processes in rats. Pharm Res 23(9):2165–2174
61. Porter CH et al (2004) Susceptibility to lipase-mediated digestion reduces the oral bioavailability of danazol after administration as a medium-chain lipid-based microemulsion formulation. Pharm Res 21(8):1405–1412
62. Dahan A, Hoffman A (2007) The effect of different lipid based formulations on the oral absorption of lipophilic drugs: The ability of in vitro lipolysis and consecutive ex vivo intestinal permeability data to predict in vivo bioavailability in rats. Eur J Pharm Biopharm 67(1):96–105
63. Sek L et al (2002) Evaluation of the in-vitro digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. J Pharm Pharmacol 54(1):29–41
64. Larsen AT, Sassene P, Müllertz A (2011) In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems. Int J Pharm 417(1–2):245–255
65. Vodovozova EL et al (2000) Antitumour activity of cytotoxic liposomes equipped with selectin ligand SiaLeX, in a mouse mammary adenocarcinoma model. Eur J Cancer (Oxf, Eng 1990) 36(7):942–949
66. Hashida M, Sato K et al (1988) Characterization of a lipophilic prodrug of 5-fluorouracil with a cholesterol promoiety and its application to liposomes. Chem Pharm Bull (Tokyo) 36(8):3186–3189
67. Sun W et al (2008) Preparation and evaluation of N3-O-toluyl-fluorouracil-loaded liposomes. Int J Pharm 353(1–2):243–250

68. Fonseca C et al (2005) Targeting of sterically stabilised pH-sensitive liposomes to human T-leukaemia cells. Eur J Pharm Biopharm 59(2):359–366
69. Roux E et al (2004) Serum-stable and long-circulating, PEGylated, pH-sensitive liposomes. J Control Release 94(2–3):447–451
70. Shi G et al (2002) Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations. J Control Release 80(1–3):309–319
71. Ferraretto A et al (1996) Characterization of biotinylated liposomes sensitive to temperature and pH: new tools for anti-cancer drug delivery. Chem Phys Lipids 82(2):133–139
72. Pradhan P et al (2010) Targeted temperature sensitive magnetic liposomes for thermo-chemotherapy. J Control Release 142(1):108–121
73. Yudina A et al (2011) Ultrasound-mediated intracellular drug delivery using microbubbles and temperature-sensitive liposomes. J Control Release 155(3):442–448
74. Zhang H et al (2011) Development and characteristics of temperature-sensitive liposomes for vinorelbine bitartrate. Int J Pharm 414(1–2):56–62
75. Hildebrand A et al (2004) Solubilization of negatively charged DPPC/DPPG liposomes by bile salts. J Colloid Interface Sci 279(2):559–571
76. Wu J et al (2007) Vascular targeting of doxorubicin using cationic liposomes. Int J Pharm 337(1–2):329–335
77. Hafez IM, Cullis PR (2000) Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. Biochim Biophys Acta 1463(1):107–114
78. Anthony AA, Mumuni AM, Philip FB (2012) Lipid nanoparticulate drug delivery systems: a revolution in dosage form design and development. In: Sezer AD (ed) Recent advances in novel drug carrier systems. Humana, Totowa
79. Allen TM et al (1995) A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim Biophy Acta 1237(2):99–108
80. Sapra P, Allen TM (2003) Ligand-targeted liposomal anticancer drugs. Prog Lipid Res 42(5):439–462
81. Patere SN, Shah SM, Pankaj OP, Nagarsenker MS (2011) Liposomal drug delivery system for receptor based hepatic targeting. In 38th annual meeting and exposition of the controlled release society. 2011, Controlled Release Society: Gaylord National Hotel, National Harbor, Maryland, USA. p. 437
82. Byrne JD, Betancourt T, Brannon-Peppas L (2008) Active targeting schemes for nanoparticle systems in cancer therapeutics. Adv Drug Deliv Rev 60(15):1615–1626
83. Chen X-Y et al (2013) Creation of lung-targeted dexamethasone immunoliposome and its therapeutic effect on bleomycin-induced lung injury in rats. PLoS One 8(3):e58275
84. Debs RJ, Heath TD, Papahadjopoulos D (1987) Targeting of anti-Thy 1.1 monoclonal antibody conjugated liposomes in Thy 1.1 mice after intraveneous administration. Biochim Biophys Acta 901(2):183–190
85. Elbayoumi TA, Torchilin VP (2007) Enhanced cytotoxicity of monoclonal anticancer antibody 2C5-modified doxorubicin-loaded PEGylated liposomes against various tumor cell lines. Eur J Pharm Sci 32(3):159–168
86. Koning GA, Kamps JAAM, Scherphof GL (2002) Efficient intracellular delivery of 5-fluorodeoxyuridine into colon cancer cells by targeted immunoliposomes. Cancer Detect Prev 26(4):299–307
87. Lukyanov AN et al (2004) Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anti-cancer antibody. J Control Release 100(1):135–144
88. Suzuki S, Uno S, Fukuda Y, Aoki Y, Masuko T, Hashimoto Y (1995) Cytotoxicity of anti-c-erbB-2 immunoliposomes containing doxorubicin on human cancer cells. Br J Cancer 72(3):663–668
89. Suzuki S et al (1995) Preparation of long-circulating immunoliposomes containing adriamycin by a novel method to coat immunoliposomes with poly(ethylene glycol). Biochim Biophys Acta 1245(1):9–16

90. Wolff B, Gregoriadis G (1984) The use of monoclonal anti-Thy1IgG1 for the targeting of liposomes to AKR-A cells in vitro and in vivo. Biochim Biophys Acta 802(2):259–273
91. Kawakami S et al (2000) Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. Biochim Biophys Acta 1524(2–3):258–265
92. Kaneo Y, Tanaka T et al (2001) Evidence for receptor-mediated hepatic uptake of pullulan in rats. J Control Release 70(3):365–373
93. Guhagarkar S et al (2011) Evaluation of pullulan-functionalized doxorubicin nanoparticles for asialoglycoprotein receptor-mediated uptake in Hep G2 cell line. Cancer Nanotechnol 2(1–6):49–55
94. Amir K, Jaleh V, Abbas Jafarian D (2012) Targeting etoposide to acute myelogenous leukaemia cells using nanostructured lipid carriers coated with transferrin. Nanotechnology 23(40):405101
95. Garanger E, Boturyn D, Dumy P (2007) Tumor targeting with RGD peptide ligands-design of new molecular conjugates for imaging and therapy of cancers. Anticancer Agents Med Chem 7(5):552–558
96. Fang B, Jiang L et al (2013) A novel cell-penetrating peptide TAT-A1 delivers siRNA into tumor cells selectively. Biochemie 95(2):251–257
97. Biswas, S., et al., Surface functionalization of doxorubicin-loaded liposomes with octa-arginine for enhanced anticancer activity. Eur J Pharm Biopharm 84(3): 517-525.
98. Banerjee R, Tyagi P et al (2004) Anisamide-targeted stealth liposomes: a potent carrier for targeting doxorubicin to human prostate cancer cells. Int J Cancer 112(4):693–700
99. Mukherjee A, Prasad TK et al (2005) Haloperidol-associated stealth liposomes: a potent carrier for delivering genes to human breast cancer cells. J Biol Chem 280:15619–15627
100. Tsuruta W et al (2009) Application of liposomes incorporating doxorubicin with sialyl Lewis X to prevent stenosis after rat carotid artery injury. Biomaterials 30(1):118–125
101. Jones MN (1994) Carbohydrate-mediated liposomal targeting and drug delivery. Adv Drug Deliv Rev 13(3):215–249
102. Mercadal M et al (1998) Preparation of immunoliposomes directed against CD34 antigen as target. Biochim Biophys Acta 1371(1):17–23
103. Yang T et al (2007) Preparation and evaluation of paclitaxel-loaded PEGylated immunoliposome. J Control Release 120(3):169–177
104. Kitagawa S, Kasamaki M, Kasamaki M (2006) Enhanced delivery of retinoic acid to skin by cationic liposomes. Chem Pharm Bull (Tokyo) 54(2):242–244
105. Knudsen NÃs et al (2011) Targeting of liposome-associated calcipotriol to the skin: effect of liposomal membrane fluidity and skin barrier integrity. Int J Pharm 416(2):478–485
106. Geusens B et al (2009) Ultradeformable cationic liposomes for delivery of small interfering RNA (siRNA) into human primary melanocytes. J Control Release 133(3):214–220
107. Goppert TM, Muller RH (2003) Plasma protein adsorption of Tween 80- and poloxamer 188-stabilized solid lipid nanoparticles. J Drug Target 11(4):225–231
108. Rezazadeh M, Emami J, Varshosaz J (2012) Cellular uptake of targeted nanostructured lipid carrier (NLC) and cytotoxicity evaluation of encapsulated paclitaxel in HT29 cancer cells. Res Pharm Sci 7(5):S191
109. Emami J et al (2012) Formulation of LDL targeted nanostructured lipid carriers loaded with paclitaxel: a detailed study of preparation, freeze drying condition, and in vitro cytotoxicity. J Nanomater 2012:10
110. Liu Y, Li K et al (2010) Folic acid conjugated nanoparticles of mixed lipid monolayer shell and biodegradable polymer core for targeted delivery of Docetaxel. Biomaterials 31(2):330–338
111. Vyas SP, Subhedar R, Jain S (2006) Development and characterization of emulsomes for sustained and targeted delivery of an antiviral agent to liver. J Pharm Pharmacol 58(3):321–326
112. Cevc G, Blume G (2001) New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, Transfersomes. Biochim Biophys Acta 1514(2):191–205

113. Elsayed MMA et al (2006) Deformable liposomes and ethosomes: mechanism of enhanced skin delivery. Int J Pharm 322(1–2):60–66
114. Cevc G, Blume G (1992) Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim Biophys Acta 1104(1):226–232
115. Cevc G (1996) Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit Rev Drug Carrier Syst 13(3–4):257–388
116. El Maghraby GMM, Williams AC, Barry BW (2000) Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. Int J Pharm 196(1):63–74
117. El Maghraby GMM, Williams AC, Barry BW (2000) Skin delivery of oestradiol from lipid vesicles: importance of liposome structure. Int J Pharm 204(1–2):159–169
118. Trotta M et al (2004) Deformable liposomes for dermal administration of methotrexate. Int J Pharm 270(1–2):119–125
119. Jain S, Sapre R et al (2005) Proultraflexible lipid vesicles for effective transdermal delivery of levonorgestrel: development, characterization, and performance evaluation. AAPS PharmSciTech 6(3):E513–E522
120. Kim A et al (2004) In vitro and in vivo transfection efficiency of a novel ultradeformable cationic liposome. Biomaterials 25(2):305–313
121. Hiruta Y et al (2006) Novel ultra-deformable vesicles entrapped with bleomycin and enhanced to penetrate rat skin. J Control Release 113(2):146–154
122. Maghraby GMME, Williams AC, Barry BW (2001) Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro. J Pharm Pharmacol 53(8):1069–1077
123. Gupta PN et al (2005) Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. Int J Pharm 293(1–2):73–82
124. Fang Y-P et al (2009) Topical delivery of 5-aminolevulinic acid-encapsulated ethosomes in a hyperproliferative skin animal model using the CLSM technique to evaluate the penetration behavior. Eur J Pharm Biopharm 73(3):391–398
125. Dubey V, Mishra D, Jain NK (2007) Melatonin loaded ethanolic liposomes: physicochemical characterization and enhanced transdermal delivery. Eur J Pharm Biopharm 67(2):398–405
126. Bhadra D et al (2004) Ethosomes: a novel vesicular carrier for enhanced transdermal delivery of an antiHIV agent. J Pharm Sci 66:72–81
127. Lopez-Pinto JM, Gonzalez-Rodreguez ML, Rabasco AM (2005) Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int J Pharm 298(1):1–12
128. Paolino D et al (2005) Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers. J Control Release 106(1–2):99–110
129. Touitou E et al (2000) Ethosomes as novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J Control Release 65(3):403–418
130. Dragicevic-Curic N et al (2009) Development of different temoporfin-loaded invasomes as novel nanocarriers of temoporfin: characterization, stability and in vitro skin penetration studies. Colloids Surf B Biointerfaces 70(2):198–206
131. Bawa R (2008) Nanoparticle-based therapeutics in humans: a survey. Nanotechnol Law Bus 5(2):135–155
132. Etheridge ML et al (2013) The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomed Nanotechnol Biol Med 9(1):1–14

Chapter 14
Nanoemulsion in Drug Targeting

Sushama Talegaonkar and Lalit Mohan Negi

14.1 Introduction

Nanoemulsions are non-equilibrium emulsions with a significantly small droplet size in the range of 20–200 nm [1]. Nanoemulsions are transparent or translucent systems (with blue hue) kinetically stable in contrast to the thermodynamically stable microemulsions. Nanoemulsions cannot be formed spontaneously and therefore requires energy input. The emulsions prepared by methods such as phase inversion temperature/composition and self-emulsifying, although having an extremely small droplet size (in nano-metric range), should not be considered as nanoemulsions, owing to the effect of the method of preparation on the droplet size, stability, and other emulsion properties [2]. Furthermore, these two systems are basically different in terms of thermodynamic stability. They remarkably differ in their behavior towards dilution and/or temperature fluctuations, i.e., the morphology and size of microemulsions are strongly affected and even destroyed by temperature changes and/or dilutions, whereas nanoemulsion droplets will remain notably stable in such conditions of stress [2]. Nanoemulsions and microemulsions are conceptually different and we are using the term nanoemulsion to the nanosize range emulsion throughout this chapter to avoid the confusion.

Nanoemulsions have been intensively exploited for their wide range of applications in drug delivery and pharmaceutics. Their versatile characteristics including enhanced drug loading capabilities, protection to the drug payload in the biological environment, increased oral bioavailability and sustain drug release makes them the suitable candidate in variety of formulation requirements. The commercial success of nanoemulsions is evident by the presence of several marketed formulations and patents for drugs of different pharmacological categories Table 14.1.

S. Talegaonkar (✉) • L.M. Negi
Department of Pharmaceutics, Jamia Hamdard, New Delhi, India
e-mail: stalegaonkar@gmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_14

Table 14.1 Commercial impact of nanoemulsions [3, 4]

Nanoemulsion based marketed formulations			
Therapeutic agent	Indication	Marketed brand	Manufacturer
Dexamethasone	Steroid	Limethason	Mitsubishi Pharmaceuticals
Propofol	Anesthetic	Diprivan	Astra Zeneca
Palmitate alprostadil	Vasodilator platelet inhibitor	Liple	Mitsubishi Pharmaceuticals
Flurbiprofen axetil	NSAID	Ropion	Kaken Pharmaceuticals
Vitamin A, D, E, K	Parenteral nutrition	Vitalipid	Fresenius Kabi, Europe
Commercial patents on nanoemulsion formulations			
Company	*Patent number*	*Patent title*	
L'Oreal (Paris, FR)	US Patent number: 5,753,241	Transparent nanoemulsion less than 100 nm based on fluid non-ionic amphiphilic lipids and use in cosmetics or in dermo pharmaceuticals	
	US Patent number: 6,689,371	Nanoemulsions based on sugar fatty ethers and its uses in cosmetics, dermatological, and/ophthalmological fields	
	Patent number: 6,464,990	Nanoemulsion based on ethylene oxide and propylene oxide block copolymers and its use in cosmetics, dermatological and ophthalmological fields	
	Patent number: 6,541,018	Nanoemulsion based on glycerol fatty esters and its uses in cosmetics, dermatological, and ophthalmological fields	
	Patent number: 6,335,022	Nanoemulsions based on oxyethylenated or non-oxyethylenated sorbitan fatty esters and its uses in cosmetics, dermatological, and ophthalmological fields	
	Patent number: 6,274,150	Nanoemulsions based on phosphoric acid fatty acid esters and its uses in cosmetics, dermatological, and ophthalmological fields	
NanoBio Corporation US	Patent number: 6,559.189 and 6,635,676	Nontoxic antimicrobial compositions and methods of use	
	Patent number: 6,506,803	Method of preventing and treating microbial infections	
ASAT AG Applied Science and Technology (Zug, CH)	PCT/EP99/08711	Nanoemulsion of 5-aminolevulinic acid	

For a drug to exert its desired pharmacological effect, it should come in contact with the desired receptor or cell organelle. However, most of the drugs from conventional immediate as well as controlled release formulations biodistribute throughout the body before reaching the actual site of action. Such an ambiguous distribution

of the drugs warrants a lot of side effects and lack of specificity. Nano drug delivery systems can provide a suitable answer to these problems by precise and specific drug targeting to the site of action [5, 6]. Since, nanoemulsions are the nanosized particles with marked potential in drug delivery, they can provide better opportunity to target drug efficiently. The surface structure as well as lipophilic characteristics of the nanoemulsion formulations can be effectively exploited to achieve local as well as systemic targeting of the drug molecules. This topic provides the basic structure, formulation of nanoemulsion systems, and current status of nanoemulsions in targeted drug delivery systems by using different approaches.

14.2 Structure of Nanoemulsion

Structurally, nanoemulsions are generally divided into oil-in-water (o/w), water-in-oil (w/o), and bicontinuous nanoemulsions. In w/o nanoemulsion, water droplets are dispersed in the continuous oil phase, whereas o/w nanoemulsion is formed when oil droplets are dispersed in the continuous aqueous phase. In systems where the amounts of water and oil are similar, a bicontinuous nanoemulsion may result. In all three types of nanoemulsions, the interface is stabilized by an appropriate combination of surfactants and/or cosurfactants. These structures are influenced both by the water to oil ratio and by the "preferred curvature" of the surfactant, which results from the interactions of the surfactant layer with the oil and water phases [7].

14.2.1 Formulation Components

A wide variety of natural and synthetic ingredients have been employed in nanoemulsion formulations. The lack of all-inclusive, non-empirical approach to nanoemulsion formulation to date is a reflection of the chemical and physicochemical diversity of the components [8]. A large number of oils and surfactants are available and can be used as components of nanoemulsion systems, but their use is limited due to toxicity, irritation potential, and unclear mechanism of action. One must choose materials that are biocompatible, non-toxic, clinically acceptable, and acts as an emulsifiers in an appropriate concentration range resulting into mild and non-aggressive nanoemulsion formulation [8]. The emphasis is, therefore given on the use of generally regarded as safe (GRAS) excipients.

14.2.1.1 Oil

Oils represent one of the most important excipients in the nanoemulsion formulation. The criterion standard for selecting the oil phase is that the drug should have high solubility in it (Table 14.2). High solubility of the drug in the oil phase will

Table 14.2 Commonly used nanoemulsion components

Oils			
Vegetable oils	Partial glycerides	Polyoxylglycerides	Polyalcohol esters
Soybean oil	Glyceryl monocaprylocaprate	Oleyl polyoxylglycerides	Propylene glycol monocaprylate
Castor oil	Glyceryl monostearate	Linoleyl polyoxylglycerides	Propylene glycol monolaurate
Cottonseed oil	Glyceryl distearate	Caprylocaproyl polyoxylglycerides	PEG-8 stearate
Palm oil	Glyceryl monooleate	Lauroyl polyoxylglycerides	Propylene glycol dicaprylic ester
Coconut oil	Glyceryl monolinoleate	Stearoyl polyoxylglycerides	
Surfactants			
Anionic	Prominent alkyl sulfates, sodium lauryl sulfate, sodium myreth sulfate, dioctyl sodium sulfosuccinate, perfluorobutanesulfonate, perfluorononanoate, perfluorooctanoate		
Cationic	Octenidine dihydrochloride, cetyl trimethylammonium bromide, cetylpyridinium chloride, benzethonium chloride, benzalkonium chloride, dioctadecyldimethylammonium bromide		
Zwitterionic	Phospholipids, cocamidopropyl hydroxysultaine, (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), cocamidopropyl betaine		
Non-ionic	Polyethoxylated tallow amine, poloxamers, polyoxyethylene glycol alkyl ethers, polyoxypropylene glycol alkyl ethers, polyoxyethylene glycol octylphenol ethers, glyceryl laurate, polyoxyethylene glycol sorbitan alkyl esters, sorbitan alkyl esters		
Cosurfactants			
Sorbitan monooleate, sorbitan monostearate, propylene glycol, propylene glycol monocaprylate (Capryol 90), 2-(2-ethoxyethoxy) ethanol (Transcutol), short chain alcohols, e.g., ethanol, propanol, butanol			

minimize the volume of the formulation to deliver the therapeutic dose of the drug. Lipophilic drugs have high solubility in the oil phase and hence formulated into o/w nanoemulsions [9]. Edible oils could offer more logical choice for the preparation of nanoemulsions, but they usually suffer with the issue of low solubility of the drug substances. Modified or hydrolyzed vegetable oils are distinctively used for this purpose as they lead to the good emulsification system with a large number of surfactants approved for oral administration and exhibit better drug solubility properties [9]. Novel semisynthetic medium chain derivatives which can be defined as amphiphilic compounds with surfactant properties, are progressively and effectively replacing the regular medium chain triglyceride oils [10, 11]. Selection of oil phase is a rational balance between its ability to solubilize the drugs and its capability to develop a nanoemulsion of desired characteristics. Mixture of oils can also be used to meet both of these requirements [12]. Long chains as well as medium chain triglyceride oils with different degrees of saturation have been used in the development of self-nanoemulsion formulations. Triglycerides are highly lipophilic and their solvent capacity for drugs is commonly a function of the effective

concentration of ester groups, thus on weight basis medium chain triglycerides have higher solvent capacity and resistance to oxidation compare to long chain triglycerides [13]. Other suitable oil phases are modified vegetable oils, digestible or non-digestible oils and fats such as soybean oil, hydrogenated soybean oil, olive oil, palm oil, oleic acid, sesame oil, peanut oil, and beeswax [13].

14.2.1.2 Surfactants

The surfactants constitute the essential part of the nanoemulsions and they reduce the interfacial tension between two liquids, i.e., lipid and aqueous phases [14]. They should also provide a flexible film that can readily deform around droplets and be of the appropriate lipophilic character to provide the correct curvature at the interfacial region for the desired nanoemulsion type, i.e., for o/w, w/o, or bicontinuous [15].

Depending on the nature of the polar group, surfactants can be categorized into four different groups: cationic, anionic, zwitterionic, and non-ionic. Cationic surfactants bear positive charge on their polar head group while anionic surfactants have a negative charge on their polar head group [16]. Zwitterionic surfactants have the ability to have both positive and negative charges, depending on the milieu in which they are placed. Non-ionic surfactants have no charge on their head group. Some of the commonly used surfactants are shown in the Table 14.2.

Combination of anionic or cationic surfactants of high HLB with a cosurfactant of lower HLB, a doubled chained surfactant of the appropriate molecular composition or a single chained nonionic surfactant of the polyethylene glycol alkyl ether type at appropriate temperature are generally used for the formulation of nanoemulsion and are effective in increasing the extent of nanoemulsion region. Employing surfactants from native origin such as phospholipids, the natural bio-friendly molecules, or alkyl polyglycosides seems to generate mild and nonaggressive nanoemulsion systems [8, 17]. Besides, polymeric surfactants such as poloxamers appear to be useful as well. Phospholipids are notable example of zwitterionic surfactants and exhibit excellent biocompatibility. The mixture of two surfactants which have the same alkyl chain length but a quite opposite solubilizing ability toward oil and water may enhance the mutual solubilization of oil and water.

Attempts have been made to rationalize surfactant behavior in terms of the hydrophilic–lipophilic balance (HLB), as well as the critical packing parameter (CPP). The HLB takes into account the relative contribution of hydrophilic and hydrophobic fragments of the surfactant molecule. It is generally accepted that low HLB (3–6) surfactants are favored for the formation of w/o nanoemulsions whereas surfactants with high HLB (8–18) are preferred for the formation of o/w nanoemulsion systems. Ionic surfactants such as sodium dodecyl sulphate that have HLB greater than 20, often require the presence of a cosurfactant to reduce their effective HLB to a value within the range required for nanoemulsion formation. Approximations of HLB for those surfactants not described by Griffin can be made either from the characterization of their water dispersibility or from an experimental estimation of their HLBs [18].

In contrast, the CPP relates to the ability of surfactants to form particular aggregates to the geometry of the molecule itself [15, 19]. The CPP can be calculated using the following equation:

$$\mathrm{CPP} = \upsilon / a \cdot \mathrm{lc}$$

υ = partial molar volume of the hydrophobic portion of surfactant.

a = optimal head group area.

lc = length of surfactant tail (critical length of the hydrophobic chain) generally assumed to be 70–80 % of its full extended length [15].

The CPP is the measure of the preferred geometry adopted by the surfactant and consequently is predictive of the type of aggregate that is likely to form. If CPP is equal to 1/3, it represents globular structure of surfactant. If it is equal to 1/2 then it represents cylindrical structure of surfactant and a value of 1 represents planer structure. Changes in nanoemulsion composition will modify the microenvironment of the surfactant, which will lead to changes in the apparent CPP of the surfactant. For example in a nanoemulsion system, penetration of small oil molecules between the hydrocarbon tails would be expected to increase the effective surfactant hydrophobe volume, whereas molecular volume oils would not be expected to exert much effect on the CPP [20].

Safety is a major determining factor in choosing a surfactant. Emulsifiers of natural origin are preferred since they are considered to be safer than the synthetic surfactants. However, these excipients have a limited nanoemulsification capacity. As a general guideline, nonionic and zwitterionic surfactants are less toxic than ionic ones. Usually the surfactant concentration ranges between 30 and 60 % v/v in order to form stable nanoemulsion. It is very important to determine the surfactant concentration properly as large amounts of surfactants may cause skin and GI irritation [11, 21, 22].

14.2.1.3 Cosurfactant

An essential requirement for the formation and stability of nanoemulsion is the attainment of a very low interfacial tension (γ). Since nanoemulsions have a very large interface between oil and water because of the small droplet size, they can only be thermodynamically stable if the interfacial tension is so low that the positive interfacial energy (given by γA, where A is the interfacial area) can be compensated by the negative free energy of mixing (Gm).

Transient negative interfacial tension is rarely achieved by the use of single surfactant, usually necessitating the addition of a co-surfactant [23–25]. Fluid interfacial film is again achieved by the addition of a co-surfactant. In the absence of co-surfactant, a highly rigid film is formed by the surfactant and thus a nanoemulsion is produced over only a very limited range of concentration. The presence of co-surfactants allows the interfacial film sufficient flexibility to take up different curvatures required to form nanoemulsion over a wide range of composition [15, 19].

The cosurfactant together with the surfactant reduce the interfacial tension down to a very small even transient negative value at which the interface would expand to form ultra small dispersed droplets, and consequently adsorb more surfactant and surfactant/cosurfactant until their bulk condition is depleted enough to give interfacial tension positive again. This process which is known as "spontaneous emulsification" forms the nanoemulsion. Thus, based on ability of the cosurfactant to affect the solvent properties of oil and/or water and to penetrate the surfactant interfacial monolayer, it can:

- Reduce further the interfacial tension. Increase the fluidity of interfaces.
- Destroy liquid crystalline and/or gel structures which prevent the nanoemulsion formation.
- Adjust HLB value and spontaneous curvature of the interface by changing surfactant partitioning characteristics.
- Decrease the sensitivity to structure fluctuations and brings formulation to its optimum state.

Medium chain length alcohols, which are commonly added as cosurfactants, have the effect of further reducing the interfacial tension, whilst increasing the fluidity of the interface thereby increasing the entropy of the system [23–25]. Medium chain length alcohols also increase the mobility of the hydrocarbon tail and also allow greater penetration of the oil into this region. It has also been suggested that some oils like ethyl esters of fatty acids, also act as cosurfactants by penetrating the hydrophobic chain region of the surfactant monolayer [20]. All of the aforementioned mechanisms are considered to facilitate nanoemulsion formation. In the case of nanoemulsions stabilized by ionic surfactants, the addition of alkanols also serves to reduce repulsive interactions between the charged head groups. Lamellar liquid crystalline phases rather than nanoemulsion phase often form with longer chain length cosurfactant or in the absence of cosurfactant due to the rigidity of the interfacial film. A wide variety of substances belonging to chemically different categories can function as cosurfactants including non-ionic surfactants [26], alcohols [27], alkanoic acids, alkanediols, and alkyl amines [28].

14.3 Method of Preparation

Nanoemulsions are prepared by different methods depending on the instrument and the mechanism involved. Some of the methods require no external forces and which depend upon their self emulsification potential which acts inherently or on application of stimuli like temperature while the other require use of brute forces to break the oil droplets sufficiently into the nano-range. The most popular machines which were developed over the period of time to prepare nano range oil emulsions are: high pressure homogenizer and microfluidizer.

14.3.1 Kinetic Methods

Kinetic methods of nanoemulsion fabrication require application of brute force. Such formulations are not thermodynamically favorable and are stabilized by the kinetic properties of colloids.

14.3.1.1 High Pressure Homogenization Technique

This technique employ, high-pressure homogenize to produce nanoemulsions of extremely low particle size. This process involves different forces, such as hydraulic shear, intense turbulence, and cavitation that act together to yield nano-sized lipid emulsions (Fig. 14.1). The ensuing nanoemulsion can be again subjected to the high-pressure homogenization until nanoemulsion with desired droplet size and polydispersity index (PDI) is obtained. The emulsion is preferably manufactured at high volume faction of the disperse lipid phase and can be diluted afterwards. However, very high phase volume ratios may result in instability by coalescence during emulsification or storage. If possible, the surfactant can be dissolved in the disperse phase instead of continuous phase; this often leads to smaller droplets. The process variable such as number of homogenization cycles as well as intensity of homogenization should be optimized carefully to achieve the desired size and PDI [29, 30].

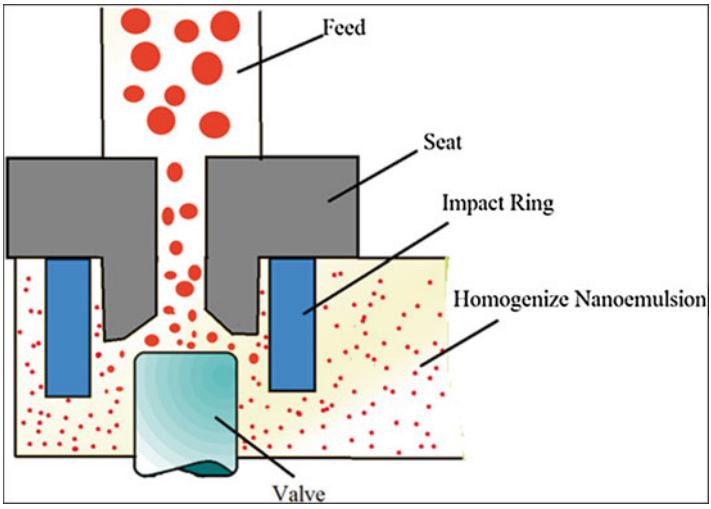

Fig. 14.1 High pressure homogenization method for the manufacture of nanoemulsions

14.3.1.2 Microfluidization Technique

Microfluidization is a patented mixing technology which uses microfluidizer device to produce extremely small size fluid particles. The microfluidizer employs high-pressure positive displacement pump (500–20,000 psi), which forces the fluid through specially designed interaction chamber, consisting of very small "micro-channels". The fluid flows through these microchannels on to an impingement area to form very fine particles of submicron range (Fig. 14.2). For the manufacture of the nanoemulsion the previously prepared coarse emulsion is introduced into a microfluidizer where it is further processed to obtain the nanoemulsion of desired specificity [3]. The process can be repeated many times to achieve the desired characteristics. The bulk emulsion can be further processed by filtration through a filter under nitrogen to remove large droplets resulting in a uniform nanoemulsion. High-pressure homogenization and microfluidization can be used for manufacture of nanoemulsions at laboratory as well as scaled up to industrial scale.

14.3.1.3 The Solvent Displacement Method

This technique is based on the nano-precipitation method used for polymeric nanoparticles. The oil phase is dissolved in water-miscible organic solvents followed by the addition of organic phase into an aqueous media containing surfactant

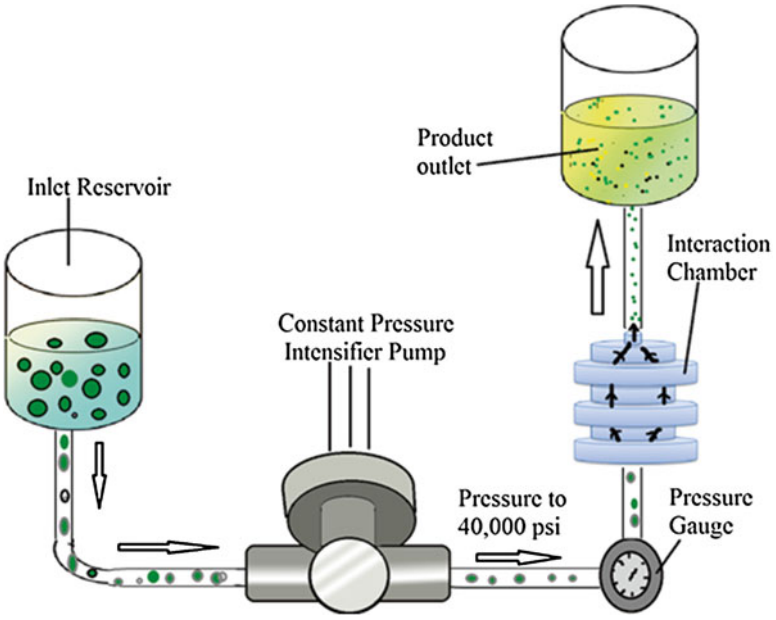

Fig. 14.2 Microfluidization method for the manufacture of nanoemulsions

to yield spontaneous nanoemulsion by rapid diffusion of organic solvent. The organic solvent is removed from the nanoemulsion by a suitable means, such as vacuum evaporation. However, no real brute force is required for the preparation of nanoemulsion by this method; still a continuous nanoemulsion is required along with the facilitation of nanonization by solvent diffusion [31]. The major limitation of this method is the use of organic solvents, such as acetone, which require additional inputs for their removal from nanoemulsion. Furthermore, a relatively dilute nanoemulsion is formed by this method which is highly undesirable [3].

14.3.2 Thermodynamic Methods

These methods lead to the formation of nanoemulsions spontaneously and are favored by thermodynamic forces. They do not require application of external force for the preparation nanoemulsions and hence are generally more stable. These systems are generally referred as microemulsion rather than nanoemulsion. However, the oil droplet size is sufficiently in nano-range and they could be considered as nanoscale emulsion.

14.3.2.1 Phase Inversion Temperature (PIT) Technique

Phase inversion temperature (PIT) technique for preparation of nanoemulsion depends upon the temperature induced HLB changes of the nonionic surfactants, such as polyethoxylated surfactants, to modify their affinities for water and oil [32]. It has been observed that polyethoxylated surfactants tend to turn lipophilic on heating due to dehydration of polyoxyethylene groups. In this method, oil, water, and nonionic surfactants are mixed together at room temperature. This mixture at room temperature forms a typical o/w microemulsion coexisting with oil, and the surfactant monolayer exhibiting positive curvature. On gradual rise in temperature macroemulsion, the polyethoxylated surfactant becomes lipophilic and at higher temperatures, the surfactant gets completely solubilized in the oily phase (Fig. 14.3). The initial o/w emulsion undergoes phase inversion to w/o emulsion through this temperature change. Electrolytes are often used in aqueous phase to lower the phase transition temperature. Multiple cycles of temperature changes results in sufficient reduction in oil droplet size and formation of nanoemulsion [3].

14.3.2.2 Self-Nanoemulsification Technique

Self-nanoemulsification method is the best known and the most preferred method as it generates nanoemulsions at room temperature without use of any organic solvent, high shear, or heat. Kinetically stable nanoemulsions with small droplet size (~50 nm) can be manufactured by the gradual addition of water into the phase

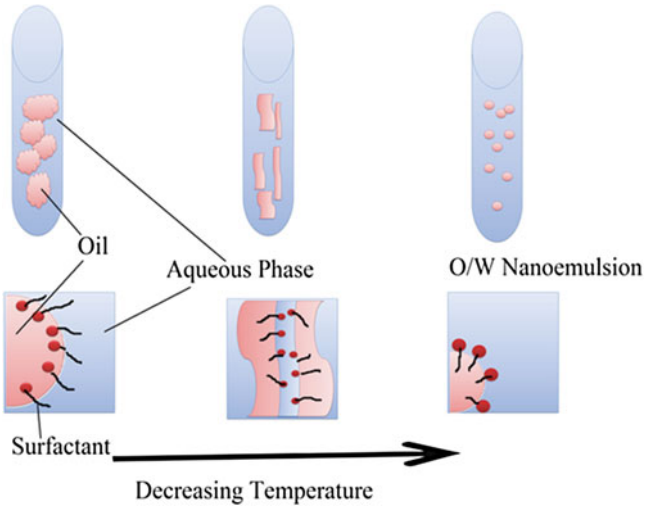

Fig. 14.3 Phase inversion temperature method for the manufacture of nanoemulsions

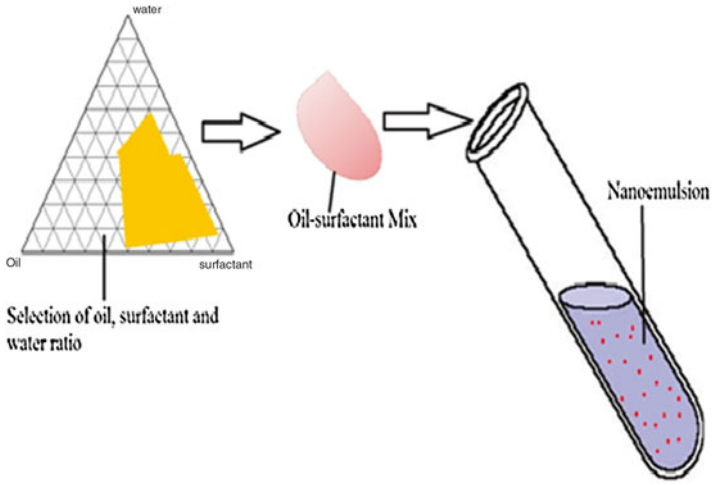

Fig. 14.4 Self-emulsification method for the manufacture of nanoemulsions

containing surfactant mix (Smix) and oil, and the nanosized emulsion is produced on gentle at constant temperature (Fig. 14.4). The spontaneous nanoemulsification has been related to the phase transitions during the emulsification process and involves lamellar liquid crystalline phases or D-type bicontinuous microemulsion during the process. The ideal mixture of oil, water and Smix are obtained from ternary phase diagram which is carefully prepared by phase titration [33]. Nanoemulsions obtained from the spontaneous nanoemulsification process are not thermodynamically stable; however, they have high kinetic energy, which accounts for their long-term colloidal stability.

14.3.2.3 Phase Inversion Composition Method

Phase inversion composition method is a useful technique to prepare kinetically stable nanoemulsions with small droplet size (~50 nm). The spontaneous nanoemulsification is associated with phase transitions during the emulsification process and involves lamellar liquid crystalline phases or D-type bicontinuous microemulsion during the process. Nanoemulsions attained from the spontaneous nanoemulsification process are not thermodynamically stable, even though they might have high kinetic energy and long-term colloidal stability [3].

14.4 Nanoemulsion for Targeted Drug Delivery

Nanoemulsions are the unique liquid based formulations which can be effectively used for local as well systemic targeting. The lipophilic nature of the O/W nanoemulsion supports its deposition in lipid rich areas such as skin and hence it can be used for local targeting. Very small size (20–80 nm) can be easily achieved with a well-optimized nanoemulsion [8]. Such an invariably small size can help to achieve targeting by enhanced permeation retention (EPR) effect. The surface of the nanoemulsion droplet can be modified suitably to attach ligands for specific receptors overexpressed in different conditions such as cancer. Lipophilic nature of the nanoemulsions also aid to exploit different anatomical pathways such as trigeminal neural and lymphatic pathways. Furthermore, the physical modifications such as incorporation of magnetite or use of surfactants responsive to sound waves can help to develop physical stimuli responsive targeted systems.

14.4.1 Local Targeting

Nanoemulsions can be exploited to target local pathologic conditions due to its exclusive physicochemical properties and distinctive interaction with body systems. The lipophilicity of nanoemulsions supports its interaction with skin milieu, and helps cross stratum corneum barrier and reside at skin site for a longer duration. The oil based components of the nanoemulsion promotes its lymphatic uptake and subsequent targeting of lymphatic system. Besides the lipophilic nature, nanoemulsion bears very small size which helps it to locally target some of the difficult sites such as lungs.

14.4.1.1 Local Targeting of Skin

Nanoemulsions are good carrier systems for the local targeting specifically skin or dermal sites. Due to high lipophilic nature the nanoemulsions can be modified to get accumulated at the cutaneous site and delivery the drug for prolonged duration.

Therefore, this system can be utilized for the localized delivery of the drug in the number of conditions such as psoriasis, infections, as cosmetics and cancers [34, 35]. Nanoemulsion systems cross the ardent stratum corneum with a number of mechanisms and remained there by careful optimization of components which could evaluated by skin permeation studies. The various mechanisms that facilitate corneum permeation include the following:

- The surfactant and cosurfactant components of the nanoemulsions may reduce the diffusional obstruction of the stratum corneum by inherent penetration enhancer's activity [36].
- Good contact with the skin surface due to small size and low surface tension [37].
- The permeation rate of the drug from nanoemulsion may be increased, as the affinity of a drug to the internal phase in nanoemulsion can be easily customized to favor partitioning into stratum corneum [38].
- A high concentration of the drug can be achieved in the nanoemulsion due to the high solubilizing capacity that might increase thermodynamic activity toward the skin [39].

Transdermal delivery of methotrexate, as an antipsoriatic drug was studied by [40] using nanoemulsion. It was observed that the nanoemulsion formulation can increase the transdermal permeation up to tenfold from a lecithin–water–PG–decanol–benzyl alcohol microemulsion compared to that from the water–PG solution alone, and threefold compared to an oil suspension. Also, mean lag time was considerably decreased with the nanoemulsion vehicle. A safe and non-irritating local topical delivery of clobetasol propionate was also achieved using nanoemulsion [34]. Clobetasol propionate affects the activity of the ATP in skin tissues and reduces the cell proliferation in the conditions like psoriasis. Although clobetasol propionate containing nanoemulsion contains more surfactant but it was more effective and safer than creams. 8-Methoxsalen, cyclosporine A, rice bran oil, and betamethasone dipropionate were also delivered topically by using nanoemulsion [41–44]. Better stratum corneum permeation and higher local concentrations were achieved by these formulations.

Infections localized to the skin area can be best treated by a delivery system which maintains the minimum inhibitory concentration of the antimicrobial agent at the target site, without overexposing the body. Peira and coworkers developed positively charged nanoscale emulsion of Miconazole nitrate (a broad spectrum antifungal agent) comprising water, 1-decanol/dodecanol (2:1, wt/wt), lecithin and/or decyl polyglucoside at different weight ratios, propylene glycol, 1,2-hexanediol and a cationic charge-inducing agent selected from stearylamine, and L-alanine benzyl ester (ALAB) and cetyltrimethylammonium bromide (CTAB). Skin accumulation of the drug from positively charged nanoemulsion was found to be nearly twofold higher than from their negatively charged equivalents [45]. The authors attributed increased accumulation due to the interaction between positive nanoemulsion systems and negatively charged skin cells. The results suggested that positively charged nanoemulsion can be used to achieve drug targeting without an associated increase in systemic absorption. Ciclopirox olamine containing nanoemulsions (nanoemulgel)

was developed for subungual onychomycosis and was found to have good permeation to the site. Furthermore, it was observed by confocal microscopy that the developed formulation enforced the persistent high concentration of the delivered moiety to the site of action when compared to simple oil or gel suspension [35].

Chemotherapeutic agents are associated with marked toxicity to other systems when enters systemic circulation. In this situation the better for treating the skin associated cancers is to deliver the drugs locally with lower systemic absorption. The hydrogels containing genistein-loaded nanoemulsions were used for similar purpose. A higher accumulation of genistein was detected into the skin from the formulation composed by medium chain triglycerides as oily core. For similar attributes, Severino and associates also developed the nanoemulsion formulations for the treatment of non-melanoma skin cancer [46]. Ligand based nanoemulsions can also be utilized for the longer retention of the topically applied formulations. Recently, Atrux-Tallau and team had used the concept of skin cell targeting with self-assembled ligand addressed nanoemulsion droplets to deliver a wide range of cosmetically active agents to the skin [47]. They utilized palmitoyl-KTTKS and asiaticoside to target skin cells (i.e., keratinocytes, fibroblasts). The concept can be executed to deliver anticancer agents in variety of skin associated cancers.

14.4.1.2 Lymphatic Targeting

The majority of orally administered drugs enter systemic circulation via absorption through the portal blood. However, the highly lipophilic compounds gain access to the systemic circulation by lymphatic transport and avoid hepatic first-pass metabolism. The anatomy of the intestinal mesenteric lymphatics is exclusive in its structure and function [48]. Lymphatic capillaries are slightly larger in diameter than blood capillaries and have a distinctive arrangement that permits interstitial fluid to flow into them but prevent it to escape back. The gut villi in the small intestine are supplied with specialized capillaries known as lacteals that drain fluid containing dietary lipids into lymphatic capillaries to the mesenteric lymph duct [48]. The presence of lipids causes the lymph to appear creamy white and this kind of lymph is referred as “chyle.” At another places lymph is clear, pale-yellow fluid. Lymph flows through this duct to the cysterni chyli and subsequently into the thoracic duct. The thoracic duct is about 38–45 cm long and is the central duct for return of lymph to blood [49]. The lymph is finally brought back together with the blood at the junction of the left jugular and the left subclavian veins [48, 49]. Due to these anatomical features, drugs successfully transported in the lymph and avoid hepatic first pass metabolism. The long chain fatty acid and monoglyceride lipids are first absorbed by enterocytes and moves to endoplasmic reticulum. The lipid undergoes reesterification and assembles into lipoprotein prior to the transport into mesenteric lymphs [50]. Hence, the in vivo conversions of lipid carriers provide a useful tool to avoid hepatic degradation and hence enhanced oral absorption of the host drugs (Fig. 14.5).

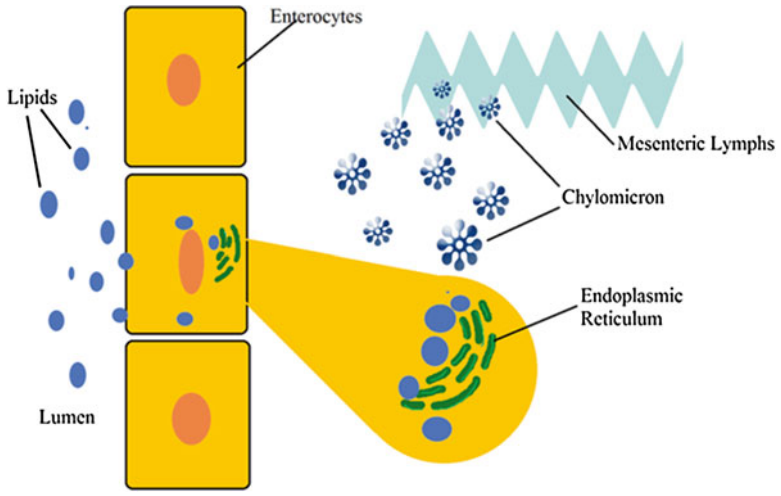

Fig. 14.5 Lymphatic absorption of lipids

The use of oil based nanoemulsion carriers is not only useful in increasing the oral bioavailability of the poorly absorbed drug but also holds prospective to design promising passive lymph-targeting drug delivery systems. A comprehensive study was conducted by Wu et al. [51] to evaluate the contribution of the lymphatic route to the intestinal absorption and transport of puerarin into the systemic circulation. The lymph duct-cannulated in unconscious rats were studied to estimate the intestinal lymphatic transport of puerarin microemulsion. The lymphatic fluid was collected at different time interval and the data was correlated with plasma concentration of puerarin. The study indicated that the lymphatic transport process contributes significantly to intestinal absorption of puerarin and hence to its systemic bioavailability. The results also suggested that the pharmaceutical scientist might use microemulsion formulations to optimize lymph-targeting drug delivery systems in various diseases.

Along with the nature of lipid used, the surfactants present in the nanoscale emulsion also affect the lymphatic uptake of the carriers [52]. The drug was determined into both lymph and plasma subsequent to the administration of micelles and microemuslion formulations containing saquinavir. The oleic acid microemulsion formulation produced higher lymph concentrations of saquinavir compared to micellar systems. The study also inferred that the nature of the surfactant used (i.e., cremophor versus D-alfa-tocopheryl polyethylene glycol 1000 succinate) appeared to alter the distribution of saquinavir between lymph and portal uptake. Earlier work with the oil based system further corroborated this fact that oil droplets can be used to target the lymphatic sites and hence the diseases with known lymphatic residence. The gelatin nanoparticles incorporated in oil emulsion were used to deliver antifilarial drugs (sodium stibogluconate). The colloidal system found to hold great potential for the purpose due to the lymphotrophicity imparted by emulsion system [53].

Marketed formulations Pirarubicin, and Lipiodol® based on emulsion carriers were also developed to target the gastric cancer and metastatic lymph nodes [54]. Hence, the natural process of lipid absorption by lymphatic site can provide an excellent opportunity to target this site by the use of nanoemulsions and treatment of various diseases originating in lymph as well as lymph nodes.

14.4.1.3 Lungs Targeting

With the increased success of nanoemulsion via nasal route in brain targeting, they were also evaluated for the targeting lungs in recent studies. Amphotericin B (AmB) nanoemulsion was developed for the delivery through Pari Sprint jet nebulizer [55] for lungs targeting. The simplicity of preparation of the AmB lipid nanoemulsions, along with their nebulization performance suggested that lipid nanoemulsion has great potential as nanocarriers for delivery of AmB to the peripheral respiratory airways. Carbamazepine nanoemulsion mist delivered by nebulizer further substantiates the utility of nanoemulsion for active targeting of poorly water-soluble compounds into the lungs [56].

Along with the drug delivery, the nanoemulsion formulations can also be used as a diagnostic agent. The folate-PFC/rhodamine nanoemulsion, with a bimodal imaging contrast agent was developed for both MRI and fluorescence imaging of nasopharyngeal carcinoma [57]. More work on the formulation and delivery device perspectives may open the interesting field of lung targeting by nanoemulsions.

14.4.2 Systemic Targeting

Systemic targeting is another mode to target wide range of organ and or organ systems. The carriers for systemic targeting often require active or passive targeting mechanism to carry the payload to the intended site. Enhanced permeation retention or EPR facilitates systemic targeting by passive mechanism while active targeting may be enforced using specific ligands or physical stimuli based release of the drug.

14.4.2.1 EPR Based Targeting

Targeted drug delivery has always been an area of fascination and possibilities along with challenges and hurdles for formulation scientists. However, despite of various method purposed to achieve this objective, EPR remained the gold standard as far the tumor targeting is concerned. It has been demonstrated previously that most solid tumors have elevated levels of vascular permeability factors such as bradykinin [58], nitric oxide [59], peroxynitrite, proteinaceous vascular permeability factor (VPF) which is equivalent to vascular endothelial growth factor [60, 61].

EPR phenomenon involves administration of long circulating nanoparticles in the systemic circulation which eventually gain access to the tumor site due to leaky vasculature. Nanoemulsion formulations were also found to have potential for the EPR mediated targeting to the tumor site. However, a careful selection is necessary for achieving the EPR targeting with such formulations. Ichikawa et al. studied the effects of the formulation components on the biodistribution of the gadolinium (Gd)-containing lipid NE (Gd-nanoLE) after its intravenous (IV) injection in D1-179 melanoma-bearing hamsters [62]. Biodistribution data revealed that Brij 700 and HCO-60 extended the retention of Gd in the blood and enhanced its accumulation in tumors. Furthermore, it was observed that soybean oil as core material yielded the highest Gd concentration in the blood and tumor, and the lowest in the liver and spleen. 10-Methoxy-9-nitrocamptothecin (MONCPT) containing nanoemulsions were prepared by using lipoid E80 and cremophor EL as main emulsifiers by microfluidization technique [63]. The developed formulation was not only found more effective against the in vitro A549 and S180 cells but also found to have greater accumulation at tumor site in the in vivo A549 cells and S180-bearing mice owing to EPR effect.

14.4.2.2 Brain Targeting

The disorders associated with the central nervous system (CNS) account for around 11 % of the global burden of disease, and these figures are likely to rise to 14 % in 2020 [4, 64]. However, a large number of newly discovered therapeutic agents have limited or no access into the CNS after administration [64, 65]. The reason for such low brain bioavailability of a number of drugs lies in the complexity of barrier system protecting it. The blood–brain barrier (BBB) is a system of vascular cellular structures, majorly constituted by tight junctions between endothelial cells, and an array of enzymes, receptors, transporters, and efflux pumps [66–68] that limit or abolish the access of chemicals of wide spectrum to the brain. These protection systems employ either the paracellular or transcellular pathways for restricting the access of molecules into the brain. A few highly lipid-soluble molecules are able to pass the BBB freely by passive diffusion from the blood to the interstitium of the brain. Chemical modifications of drug molecules with an intension to enhance their lipophilicity may result in an increased distribution in the brain. However, such modifications are arduous and often the modified molecule is treated as a new chemical entity, necessitating the comprehensive evaluation of the toxicity and efficacy testing. A nanocarrier system may, however, provide a logical and less cumbersome solution for this problem.

Nanoemulsion is one such carrier system, which can be utilized to achieve the therapeutic concentration of the drugs across the BBB. It can be administered directly into the systemic circulation [69, 70] or can be administered by using nose to brain pathway [71–73]. However some studies also, suggested that such nanoemulsion also be administered orally to enhance bioavailability as well as brain accumulation [74]. The nanoemulsion formulation developed from flax-seed oil

carrying saquinavir (an anti-HIV protease inhibitor) was studied by administering through oral as well as intravenous route [74]. A fivefold enhancement in Cmax and threefold increase in overall AUC of the drug in brain was observed in comparison to drug suspension. A drug with a potential indication in the pathological condition located in the brain and probable toxic outcomes when present in systemic circulation can possibly be rendered less toxic and more potent by altering the normal pharmacokinetic distribution. An enhanced allocation of such drugs in brain may reduce their toxic potential. A nanoemulsion formulation containing docetaxel (DNE) was compared for its relative safety as well as activity against the marketed formulation, Taxotere®. The outcomes of the study revealed that the anti-proliferation activity of the DNE was equivalent to the Taxotere® in both U87 and bEnd.3 glioma cells, but a remarkable reduction in in vivo toxicity was observed in case of DNE.

Intranasal administration of nanoemulsion is an alternate strategy to target the brain. A number of pathways including transcellular, paracellular, olfactory, and trigeminal neural pathways are responsible for the transport of a range of chemical entities to the brain by this route [71]. Several studies in rodents have demonstrated that the direct nose-to-brain transport of small molecular weight drugs is enhanced by using simple as well as surface modified nanoemulsion formulation. An added advantage of surface modification of these carriers provides mucoadhesion and enhances the effectiveness of nanoemulsions for nose to brain delivery. Risperidone and olanzapine were found to have greater accumulation in brain when delivered by nose to brain route [73, 75]. Similar observations were reported with tacrine, an anti-Alzheimer's agent [76]. The brain bioavailability of tacrine after intranasal administration of mucoadhesive was found to be twofold higher when compared with plain intravenous tacrine solution indicating larger extent of distribution of the drug to brain with intranasal mucoadhesive microemulsion. The results were further corroborated by the scintigraphy studies of the rabbit brain. Surface modification was beneficial in enhancing the safety of the nanoemulsion system. The histopathology investigation with amiloride loaded mucoadhesive nanoemulsion formulation on nasal mucosa showed lack of toxicity and integrity of granular cellular structure [77].

14.4.2.3 Ligand Based Targeting

An active targeting of cells or tissues chiefly includes ligand based targeting. Ligands include any chemical entity that identify and binds to target site antigen (mostly receptors) overexpressed or selectively expressed by particular cells or tissue sections. An ideal ligand for targeted drug delivery should have high specificity and affinity for binding to the cell surface receptors and should stimulate subsequent internalization of payload that need internalization for its intracellular action [78, 79]. They should be compatible as well as have tendency for chemical conjugation [80, 81]. Nano drug delivery systems provide excellent opportunity to modify their surfaces to develop ligand decorated delivery systems and hence can also be actively targeted to the tumor site using ligand based strategy. Some of the receptors that

Table 14.3 Various receptors for ligand based drug targeting

Receptor	Targeting therapy	Reference
HER-2	Monoclonal antibodies like Trastuzumab, Pertuzumab	[82]
EGFR	EGFR inhibitors like Geftinib, Erlotinib, Cetuximab	[83]
HER1/HER2	Dual inhibitors like Lepatinib, Neratinib	[84]
mTOR Protein kinase	Inhibitors like Everolimus, Temsirolimus	[85]
VEGF	Monoclonal antibody like Bevacizumab	[86]
IGF-1	Monoclonal antibody like CP-751871	[82]
CD44	Hyaluronan bioconjugates	[87]
PSMA receptor	Quantum dots	[88]
Transferrin receptor	siRNA	[89]
epCAM	siRNA	[90]
αvβ3/5Integrin	Pt (IV) prodrug	[91]
Collagen IV	Anti-inflammatory peptide	[92]
Fibronectin	Peptide	[93]
Folic acid	Paclitaxel and yittrium-90	[94]

have attracted the significant attention in recent time for the ligand based targeting are summarized in Table 14.3.

Aclacinomycin A is an anthracycline antitumor drug. The folate-PEG linked nanoemulsion of the aclacinomycin A was developed to target KB nasopharyngeal cells in mice xenograft [95]. A better efficacy was observed by the use of folate-PEG linked nanoemulsion in tumor regression studies as compared to the non-liganded counterpart. Talekar and associates evaluated the ceramide containing nanoemulsions with folate and EGFR receptor targeting capabilities [96]. The study showed that the targeted system improved cytotoxicity of PIK75 compared to the non-targeted system in SKOV cancer cell lines. Similarly lycorine loaded mannosylated nanoemulsion (M-LYC-OA-LNEs) was also developed and evaluated for tumor targeting. (M-LYC-OA-LNEs) was found to have potential for both drug delivery and diagnostics. Such, systems provides great value as theranostic tools. Previously Tiwari, Tan, and Amiji also developed nanoemulsion with theranostic potential [97]. This nanoemulsion system was developed for drug delivery as well as MR imaging. Thus, nanoemulsion can be used for ligand based drug targeting like other nanoparticulate systems. However, unlike other systems nanoemulsions provide greater encapsulation capacity for water insoluble drugs, are easy to develop, and form stable systems.

14.4.2.4 Physical Targeting of Nanoemulsions

In over a decade's time biocompatible magnetic fluids emerged as the interesting material in biological application [98]. Magnetic fluids comprise of magnetic nanoparticles, dispersed as stable colloids in physiological medium. This material can

be engineered for a variety of purposes including external magnetic field guided targeting [99] as well as induction of local hyperthermia of biological tissues when exposed to an AC magnetic field [100]. Hyperthermia was assumed to have cytotoxic effects on cancer cells and hence can be used as a suitable alternative for the chemotherapy.

Formulations based on nanoemulsion have several appealing characteristics such as, enhanced drug solubilization, biocompatibility, stability and ease in scale up. Collaborated properties of nanoemulsions and magnetic nanoparticles can help in development of an ideal nanocarrier system with versatile properties for drug delivery, hyperthermia induced self-cytotoxicity, targeting, and diagnosis. Researchers are working on the association of these two systems to develop delivery systems with ideal characteristics. Bakandritsos et al. proposed a simple method to prepare magnetically guided lipid based nanoemulsion by self-emulsification method [101]. Magnetic nanoparticles were synthesized separately and added to the oil-surfactant self-nanoemulsifying mixture, which on self-emulsification in water delivers magnetic nanoemulsions. Such methods can simplify the preparation of stable nanoemulsions which are magnetically active. Magnetic nanoemulsions have also found their application in photodynamic therapy (PDT) of skin cancer. Foscan®, a new generation photosensitizer was incorporated into magnetic nanoemulsion and studied for its activity in skin cancer [102]. Due to the use of nanoemulsion as the carrier, the developed system accumulated in the deeper layers of the skin and focused the activity of magnetically induced hyperthermia and/or Foscan induced light photosensitization to the site where it was needed. Though the methods for assimilation of magnetic nanoparticles into nanoemulsion have been studied, the in vivo potentials for the magnetic field induced controlled movement of magnetic nanoparticles are still to be studied comprehensively.

Sound is another physical stimulus which can be used in targeted drug delivery. The ultrasonic stimulus of specific frequency may leads to control the release of the drug from the nanocarrier system to achieve the higher concentrations at desired site. Camptothecin based perfluorocarbon nanoemulsions were developed using liquid perfluorocarbons and coconut oil as the cores of the inner phase and phospholipids and/or Pluronic F68 as stablizers. The nanoemulsions were prepared with high drug loading (approximately 100 %) with a mean droplet diameter of 220–420 nm [103]. An excellent uptake was observed in melanomas and ovarian cancer cells by confocal microscopy. One of the interesting findings of this work includes that there was an enhanced release of camptothecin from the developed system on application of 1 MHz ultrasound. Such characteristics of the acoustically stimulated nanoemulsions can be utilized to control the release of the host moiety at target site. Rapoport and co-workers also developed a novel ultrasound-mediated chemotherapeutic modality derived from systemic injections of drug-loaded nanoemulsions get converted to microbubbles in situ on application of therapeutic ultrasound. This modality proved very effective in inducing dramatic tumor regression in ovarian and pancreatic cancer models [104].

14.5 Diagnostic Applications of Nanoemulsion

An early and accurate diagnosis is a hallmark of any good treatment strategy. The diagnosis of various disease conditions in their early phase of pathology may warrant greater chances of cure and recovery. Computer tomography or CT scan is one such diagnostic tool which helps in numerous conditions to diagnose the anatomical anomaly. CT scan employs computer-processed X-rays to produce tomographic images (virtual "slices") of specific areas of the scanned portion without surgical intervention [105]. However, soft living tissues are of very close densities, which make it difficult to distinguish clearly between them. Hence, it is necessary to use a contrast media (generally the heavy elements, like iodine or barium) which allow significant augmentation of the differences in contrast between anatomic sections [106]. One of the problems with the advanced diagnostic methods like MicroCT lies in the fact that requires more than 10 min for full scan. On the other hand most of the contrast medium get cleared very rapidly providing insignificant time for a complete scan [107]. Therefore, a contrast medium with long circulation properties is an utmost requirement for such procedures. The nanoemulsion containing iodinated oil and with PEGylated surface can provide distinct solution for this problem [108]. After intravenous injection, the homogeneous biodistribution of Fenestra® (An iodinated nanoemulsion) in the blood is achieved within 10 min [109]. In mice, the contrast effect it in the blood pool remaining after injection is of around 1 h for normal nanoemulsions, which can be exceeded to 2 h for PEGylated form [109, 110]. In rabbits, PEGylated nanoemulsions still circulate in the blood pool for up to 3 h [111]. However, there might be many formulation related aspects which may influence the performance of nanoemulsion as contrast carrier. Hallouard et al. showed that the free surfactant material significantly affect the pharmacokinetics of the nanoemulsion contrast media [112]. Similarly toxicity of such carriers could be a reason of concern in use of such contrast carriers. Much safer nanoemulsion contrast carriers are under exploration for abolishing such toxicological apprehensions [113].

14.6 Conclusion

Nanoemulsion drug delivery system is a multipurpose formulation to deliver a wide variety of payloads in a variety of conditions. Due to the choice of huge number of core materials as well as emulsifying surface components offered by nanoemulsions they can be customized to cater different needs. Targeting is one of the desired in a variety of conditions to achieve the necessary effect at the required location and avoid the toxicity at other sites. Nanoemulsion drug delivery systems can be tailored to achieve both active ligand based as well as passive EPR dependant targeting. They can also utilize to deliver the drug at local sites (skin) or can be used to attain organ specific targeting such as brain and lungs. Apart from cells and organs

nanoemulsions can also achieve organ system targeting (lymphatic system) due to their inherent chemical properties and biological disposition. Furthermore, the localization as well as release of drug from the new generation target based nanoemulsions can be controlled external physical stimuli. Therefore, nanoemulsion drug delivery system is truly a versatile tool for achieving desired drug targeting in a variety of conditions.

References

1. Solans C, Izquierdo P, Nolla J, Azemar N, Garcia-Celma MJ (2005) Nano-emulsions. Curr Opin Colloid Interface Sci 10:102–110
2. Anton N, Vandamme TF (2011) Nano-emulsions and micro-emulsions: clarifications of the critical differences. Pharm Res 28:978–985
3. Lovelyn C, Attama AA (2011) Current state of nanoemulsions in drug delivery. J Biomater Nanobiotechnol 2:626–639
4. Tiwari SB, Amiji MM (2006) Nanoemulsion formulations for tumor-targeted delivery. In: Amiji MM (ed) Nanotechnology for cancer therapy. Taylor and Francis Group, New York, pp 723–39
5. Sahoo S, Labhasetwar V (2003) Nanotech approach to drug delivery and imaging. Drug Discov Today 8:1112–1120
6. Mehra NK, Mishra V, Jain NK (2013) Receptor-based targeting of therapeutics. Ther Deliv 4:369–394
7. Biasis J, ClinB LP (1987) Microemulsions: Structure and Dynamics. CRC, Boca Raton
8. Azeem A, Khan ZI, Aqil M, Ahmad FJ, Khar RK, Talegaonkar S (2009) Microemulsions as a surrogate carrier for dermal drug delivery. Drug Dev Ind Pharm 35:525–547
9. Talegaonkar S, Akhter S, Jain GK, Ahmad FJ, Khar RK, Jain N, Khan ZI (2008) Investigation of nanoemulsion system for transdermal delivery of domperidone: ex-vivo and in vivo studies. Curr Nanosci 4:381–390
10. Karim A, Gokhale R, Cole M, Sherman J, Yeramian P, Bryant M, Franke H (1994) HIV protease inhibitor SC-52151: a novel method of optimizing bioavailability profile via a microemulsion drug delivery system. Pharm Res 11:S368
11. Constantinides PP (1995) Lipid microemulsions for improving drug dissolution and oral absorption and biopharmaceutical aspects. Pharm Res 12:1561–1572
12. Jumaa M, Mueller BW (2002) Formulation and stability of benzodiazepines in a new lipid emulsion formulation. Pharmazie 57:740–743
13. Anderson BD (1999) Chemical and related factors controlling lipid solubility. Bull Tech Gatt 92:11–18
14. Monsteqszia A, Haqqueniq S (2012) Surfactant science and technology. Wiley, New Jersey
15. Lawrence MJ, Rees GD (2000) Microemulsion-based media as novel drug delivery systems. Adv Drug Deliv Rev 45:89–121
16. Hait SK, Moulik SP (2002) Gemini surfactants: a distinct class of self-assembling molecules. Curr Sci 82:1101–1111
17. Ananthapadmanabhan KP et al (2009) A novel technology in mild and moisturizing cleansing liquids. Cosmet Dermatol 22:307–316
18. Griffin WC (1954) Calculation of HLB values of non ionic surfactants. J Soc Cosmet Chem 5:249–256
19. Ghosh PK, Murthy RSR (2006) Microemulsions: a potential drug delivery system. Curr Drug Deliv 3:167–180

20. Warisnoicharoen W, Lansley AB, Lawrence MJ (2000) Nonionic oil-in-water microemulsions: the effect of oil type on phase behavior. Int J Pharm 198:7–27
21. Yuasa H, Sekiya M, Ozeki S, Watanabe J (1994) Evaluation of milk fat globulemembrane (MFGM) emulsion for oral administration: absorption of α-linolenic acidin rats and the effect of emulsion droplet size. Biol Pharm Bull 17:756–758
22. Hauss DJ, Fogal SE, Ficorilli JV, Price CA, Roy T, Jayaraj AA, Keirns JJ (1998) Lipid-based delivery systems for improving the bioavailability and lymphatic transport of a poorly water-soluble LTB4 inhibitor. J Pharm Sci 87:164–169
23. Attwood D, Mallon C, Taylor CJ (1992) Phase studies on oil-in-water phospholipid microemulsions. Int J Pharm 84:R5–R8
24. Eccleston J (1994) Microemulsions. In: Swarbrick J, Boylan JC (eds) Encyclopedia of pharmaceutical technology. Marcel Dekker, New York, pp 375–421
25. Lawrence MJ (1996) Microemulsions as drug delivery vehicles. Curr Opin Colloid Interface Sci 1:826–832
26. OsborneDW MCA, Rogers RL (1988) Alcohol-free microemulsions. J Dispers Sci Technol 9:415–423
27. Aboofazeli R, Patel N, Thomas M, Lawrence MJ (1995) Investigations into the formation and characterisation of phospholipid microemulsions. IV. Pseudo-ternary phase diagrams of systems containing water–lecithin–alcohol and oil; the influence of oil. Int J Pharm 125:107–116
28. Aboofazeli R, Lawrence CB, Wicks SR, Lawrence MJ (1994) Investigations into the formation and characterisation of phospholipid microemulsions. III. Pseudo-ternary phasediagrams of systems containing water–lecithin–isopropylmyristate and either an alkanoic acid, amine, alkanediol,polyethylene glycol alkyl ether or alcohol as cosurfactant. Int J Pharm 111:63–72
29. Bouchemal K, Briancon S, Fessi H, Perrier E (2004) Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimization. Int J Pharm 280:241–251
30. Musa SH, Basri M, Masoumi HR, Karjiban RA, Malek EA, Basri H, Shamsuddin AF (2013) Formulation optimization of palm kernel oil esters nanoemulsion-loaded with chloramphenicol suitable for meningitis treatment. Colloids Surf B Biointerfaces 112:113–119
31. Chakraborty S, Shukla D, Vuddanda PR, Mishra B, Singh S (2010) Utilization of adsorption technique in the development of oral delivery system of lipid based nanoparticles. Colloids Surf B Biointerfaces 81:563–569
32. Marszall L (1987) HLB of nonionic surfactants: PIT and EIP methods. In: Schick MJ (ed) Nonionic surfactant: physical chemistry. Marcel Dekker, New York, pp 493–547
33. Shafiq-un-Nabi S, Shakeel F, Talegaonkar S, Ali J, Baboota S, Ahuj A, Khar RK, Ali M (2007) Formulation development and optimizationusing nanoemulsion technique: a technical note. AAPS Pharm Sci Tech 8:E1–E6
34. Alam MS, Ali MS, Alam N, Siddiqui MR, Shamim M, Safhi MM (2013) In vivo study of clobetasol propionate loaded nanoemulsion for topical application in psoriasis and atopic dermatitis. Drug Invent Today 5:8–12
35. Kumar S, Talegaonkar S, Negi LM, Iqbal Z (2013) Design and development of ciclopirox topical nanoemulsion gel for the treatment of subungual onychomycosis. Indian J Pharm Educ Res 46:303–314
36. Rhee YS, Choi JG, Park ES, Chi SC (2001) Transdermal delivery of ketoprofen using microemulsions. Int J Pharm 228:161–170
37. Elena P, Paola S, Maria RG (2001) Transdermal permeation of apomorphine through hairless mouse skin from microemulsions. Int J Pharm 226:47–51
38. Kreilgaard M (2002) Influence of microemulsion on cutaneous drug delivery. Adv Drug Deliv Rev 54:S77–S98
39. Hua L, Weisan P, Jiayu L, Ying Z (2004) Preparation, evaluation and NMR characterization of vinpocetine microemulsion for transdermal delivery. Drug Dev Ind Pharm 30:657–666
40. Trotta M, Pattarino F, Gasco MR (1996) Influence of counter ions on the skin permeation of methotrexate from water–oil microemulsion. Pharm. Acta Helv. 71:135–140

41. Baroli B, Lopez-Quintela MA, Delgado-Charro MB, Fadda AM, Blanco-Mendez J (2000) Microemulsions for topical delivery of 8-methoxsalen. J Control Release 69:209–218
42. Liu H, Li S, Wang Y, Han F, Dong Y (2006) Bicontinuous water-AOT/Tween 85-isopropyl myristate microemulsion: a new vehicle for transdermal delivery of cyclosporine A. Drug Dev Ind Pharm 32:549–557
43. Bernardi DS, Pereira TA, Maciel NR, Bortoloto J, Viera GS, Oliveira GC, Rocha-Filho PA (2011) Formation and stability of oil-in-water nanoemulsions containing rice bran oil: in vitro and in vivo assessments. J Nanobiotechnol 9:1–9
44. Alam MS, Ali MS, Alam N, Alam MI, Anwer T, Imam F, Shamim M (2012) Design and characterization of nanostructure topical gel of betamethasone dipropionate for psoriasis. J Appl Pharma Sci 2:148–158
45. Peira E, Carlotti ME, Trotta C, Cavalli R, Trotta M (2008) Positively charged microemulsions for topical application. Int J Pharm 346:119–123
46. Severino P, Fangueiro JF, Ferreira SV, Basso R, Chaud MV, Santana MH, Rosmaninho A, Souto EB (2013) Nanoemulsions and nanoparticles for non-melanoma skin cancer: effects of lipid materials. Clin Transl Oncol 15:417–424
47. Atrux-Tallau N, Delmas T, Han SH, Kim JW, Bibette J (2013) Skin cell targeting with self-assembled ligand addressed nanoemulsion droplets. Int J Cosmet Sci 35:310–318
48. O'Driscoll CM (2002) Lipid-based formulations for intestinal lymphatic delivery. Eur J Pharm Sci 15:405–415
49. Tortora GJ, Grabowski SR (2000) Introduction to the human body: the essentials of anatomy and physiology. Wiley, New York
50. Kiyasu JY, Bloom B, Chaikoff IL (1952) The portal transport of absorbed fatty acids. J Biol Chem 199:415–419
51. Wu H, Zhou A, Lu C, Wang L (2011) Examination of lymphatic transport of puerarin in unconscious lymph duct-cannulated rats after administration in microemulsion drug delivery systems. Eur J Pharm Sci 42:348–353
52. Griffin BT, O'Driscoll CM (2006) A comparison of intestinal lymphatic transport and systemic bioavailability of saquinavir from three lipid-based formulations in the anaesthetized rat model. J Phama Pharmacol 58:917–925
53. Karajgi JS, Vyas SP (1994) A lymphotropic colloidal carrier system for diethylcarbamazine: preparation and performance evaluation. J Microencapsul 11:539–545
54. Yoshimura K, Nunomura M, Takiguchi N, Oda K, Suzuki H, Furukawa R, Sarashina H, Kohda K, Saito N, Sugaya Y, Tiku T, Wakathuki K, Ishikawa H, Yasutomi J, Nakajima N (1996) Evaluation of endoscopic pirarubicin-Lipiodol emulsion injection therapy for gastric cancer. Gan To Kagaku Ryoho 23:1519–1522
55. Nasr M, Nawaz S, Elhissi A (2012) Amphotericin B lipid nanoemulsion aerosols for targeting peripheral respiratory airways via nebulization. Int J Pharm 436(1):611–616
56. Nesamony J, Kalra A, Majrad MS, Boddu SH, Jung R, Williams FE, Schnapp AM, Nauli SM, Kalinoski AL (2013) Development and characterization of nanostructured mists with potential for actively targeting poorly water-soluble compounds into the lungs. Pharm Res 30:2625–2639
57. Bae PK, Jung J, Lim SJ, Kim D, Kim SK, Chung BH (2013) Bimodal perfluorocarbon nanoemulsions for nasopharyngeal carcinoma targeting. Mol Imaging Biol 15:401–410
58. Maeda H, Matsumura Y, Kato H (1988) Purification and identification of [hydroxypropyl3] bradykinin in ascitic fluid from a patient with gastric cancer. J Biol Chem 263:16051–16054
59. Maeda H, Noguchi Y, Sato K, Akaike T (1994) Enhanced vascular permeability in solid tumor is mediated by nitric oxide and inhibited by both new nitric oxide scavenger and nitric oxide synthase inhibitor. Jpn J Cancer Res 85:331–334
60. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985
61. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309

62. Ichikawa H, Watanabe T, Tokumitsu H, Fukumori Y (2007) Formulation considerations of gadolinium lipid nanoemulsion for intravenous delivery to tumors in neutron-capture therapy. Curr Drug Deliv 4:131–140
63. Han M, He CX, Fang QL, Yang XC, Diao YY, Xu DH, He QJ, Hu YZ, Liang WQ, Yang B, Gao JQ (2009) A novel camptothecin derivative incorporated in nano-carrier induced distinguished improvement in solubility, stability and anti-tumor activity both in vitro and in vivo. Pharm Res 26:926–935
64. Pardridge WM (2007) Blood-brain barrier delivery. Drug Discov Today 12:54–61
65. Pardridge WM (2002) Drug and gene targeting to the brain with molecular Trojan horses. Nat Rev Drug Discov 1:131–139
66. Begley DJ, Brightman MW (2003) Structural and functional aspects of the blood–brain barrier. Prog Drug Res 61:39–78
67. Nies AT (2007) The role of membrane transporters in drug delivery to brain tumors. Cancer Lett 254:11–29
68. Deeken JF, Löscher W (2007) The blood–brain barrier and cancer: transporters, treatment, and Trojan horses. Clin Cancer Res 13:1663–1674
69. Gaoe H, Pang Z, Pan S, Cao S, Yang Z, Chen C, Jiang X (2012) Anti-glioma effect and safety of docetaxel-loaded nanoemulsion. Arch Pharm Res 35:333–334
70. Yao J, Zhou JP, Ping QN (2007) Characteristics of nobiletin-loaded nanoemulsion and its in vivo distribution in mice. Yao Xue Xue Bao 42:663–668
71. Talegaonkar S, Mishra P (2004) intranasal delivery: an approach to bypass the blood brain barrier. Indian J Pharmacol 36:140–147
72. Bahadur S, Pathak K (2012) Buffered nanoemulsion for nose to brain delivery of ziprasidone hydrochloride: preformulation and pharmacodynamic evaluation. Curr Drug Deliv 9:596–607
73. Kumar M, Misra A, Babbar AK, Mishra AK, Mishra P, Pathak K (2008) Intranasal nanoemulsion based brain targeting drug delivery system of risperidone. Int J Pharm 358:285–291
74. Vyas TK, Shahiwala A, Amiji MM (2008) Improved oral bioavailability and brain transport of Saquinavir upon administration in novel nanoemulsion formulations. Int J Pharm 347:93–110
75. Kumar M, Misra A, Pathak K (2009) Formulation and characterization of nanoemulsion of olanzapine for intranasal delivery. PDA J Pharm Sci Technol 63:501–511
76. Jogani VV, Shah PJ, Mishra P, Mishra AK, Misra AR (2008) Intranasal mucoadhesivemicroemulsion of tacrine to improve brain targeting. Alzheimer Dis Assoc Disord 22:116–124
77. Jain N, Akhter S, Jain GK, Khan ZI, Khar RK, Ahmad FJ (2011) Antiepileptic intranasal Amiloride loaded mucoadhesive nanoemulsion: development and safety assessment. J Biomed Nanotechnol 7:142–143
78. Murphy EA, Majeti BK, Barnes LA et al (2008) Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. Proc Natl Acad Sci U S A 105:9343–9348
79. Das M, Mohanty C, Sahoo SK (2009) Ligand-based targeted therapy for cancer tissue. Expert Opin Drug Deliv 6:285–304
80. Yukio K, Takeshi S, Takashi K et al (1996) Kinetic analysis of receptor-mediated endocytosis (RME) of proteins and peptides: use of RME as a drug delivery system. J Control Release 39:191–200
81. Sinha R, Kim GJ, Nie S et al (2006) Nanotechnology in cancer therapeutics: bioconjugated nanoparticles for drug delivery. Mol Cancer Ther 5:1909–1917
82. Vora T, Azambuja ED, Awada A, Piccart M (2009) Novel therapeutics in breast cancer: looking to the future. Update Cancer Ther 3:189–205
83. Alvarez RH, Valero V, Hortobagyi GN (2010) Emerging targeted therapies for breast cancer. J Clin Oncol 28:3366–3379.
84. Lee RJ, Armstrong AC, Wardley AM (2013) Emerging targeted combinations in the management of breast cancer. J Breast Cancer Targets Ther 14:505–515

85. Shaw RJ, Cantley LC (2006) Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 441:424–430
86. Shih T, Lindley C (2006) Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther 28:1779–1802
87. Negi LM, Talegaonkar S, Jaggi M (2012) Role of CD44 in tumour progression and strategies for targeting. J Drug Target 20:561–573
88. Gao X, Cui Y, Levenson RM, Chung LWK, Nie S (2004) In vivo cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 22:969–976
89. Bartlett DW, Su H, Hildebrandt IJ, Weber WA, Davis ME (2007) Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging. Proc Natl Acad Sci U S A 104:15549–15554
90. Winkler J, Martin-Killias P, Pluckthun A, Zangemeister-Wittke U (2009) EpCAM targeted delivery of nanocomplexed siRNA to tumor cells with designed ankyrin repeat proteins. Mol Cancer Ther 8:2674–2683
91. Graf N, Bielenberg DR, Kolishetti N, Muus C, Banyard J, Farokhzad OC, Lippard SJ (2012) αVβ3 Integrin-targeted PLGA-PEG nanoparticles for enhanced anti-tumor efficacy of a Pt(IV) prodrug. ACS Nano 6:4530–4539
92. Kamaly N, Fredman G, Subramanian M, Gadde S, Pesic A, Cheung L, Fayad ZA, Langer R, Tabas I, Farokhzad OC (2013) Development and in vivo efficacy of targeted polymeric inflammation-resolving nanoparticles. Proc Natl Acad Sci U S A 110:6506–6511
93. Saw PE, Kim S, Lee IH, Park J, Yu M, Lee J, Kim JI, Jon S (2013) Aptide-conjugated liposome targeting tumor-associated fibronectin for glioma therapy. J Math Chem B 1:4723–4726
94. Werner ME, Karve S, Sukumar R, Cummings ND, Copp JA, Chen RC, Zhang T, Wang AZ (2011) Folate-targeted nanoparticle delivery of chemo- and radiotherapeutics for the treatment of ovarian cancer peritoneal metastasis. Biomaterials 32:8548–8554
95. Ohguchi Y, Kawano K, Hattori Y, Maitani Y (2008) Selective delivery of folate-PEG-linked, nanoemulsion-loaded aclacinomycin A to KB nasopharyngeal cells and xenograft: effect of chain length and amount of folate-PEG linker. J Drug Target 16:660–667
96. Talekar M, Ganta S, Singh A, Amiji M, Kendall J, Denny WA, Garg S (2010) Phosphatidylinositol 3-kinase inhibitor (PIK75) containing surface functionalized nanoemulsion for enhanced drug delivery, cytotoxicity and pro-apoptotic activity in ovarian cancer cells. Pharm Res 29:2874–2886
97. Tiwari S, Tan Y, Amiji M (2006) Preparation and in vitro characterization of multifunctional nanoemulsions for simultaneous mr imaging and targeted drug delivery. J Biomed Nanotechnol 2:217–224
98. Watanabe M, Yoneda M, Morohashi A, Hori Y, Okamoto D, Sato A, Kurioka D, Nittami T, Hirokawa Y, Shiraishi T, Kawai K, Kasai H, Totsuka Y (2013) Effects of Fe_3O_4 magnetic nanoparticles on A549 cells. Int J Mol Sci 14:15546–15560
99. Hu SH, Hsieh TY, Chiang CS, Chen PJ, Chen YY, Chiu TL, Chen SY (2013) Surfactant-free lipo-polymersomes stabilized by iron oxide nanoparticles/polymer interlayer for synergistically targeted and magnetically guided gene delivery. Adv Healthc Mater 3:273–282. doi:10.1002/adhm.201300122
100. Huang HS, Hainfield JF (2013) Intravenous magnetic nanoparticle cancer hyperthermia. Int J Nanomedicine 8:2521–2532
101. Bakandritsos A, Zboril R, Bouropoulos N, Kallinteri P, Favretto ME, Parker TL, Mullertz A, Fatouros DG (2010) The preparation of magnetically guided lipid based nanoemulsions using self-emulsifying technology. Nanotechnology 21:055104–055112
102. Primo FL, Macaroff PP, Lacava ZGM, Azevedo RB, Morais PC, Tedesco AC (2007) Binding and photophysical studies of biocompatible magnetic fluid in biological medium and development of magnetic nanoemulsion: a new candidate for cancer treatment. J Magn Magn Mater 310:2838–2840

103. Fang JY, Hung CF, Hua SC, Hwang TL (2009) Acoustically active perfluorocarbon nanoemulsions as drug delivery carriers for camptothecin: drug release and cytotoxicity against cancer cells. Ultrasonics 49:39–46
104. Rapoport NY, Kennedy AM, Shea JE, Scaife CL, Nam KH (2009) Controlled and targeted tumor chemotherapy by ultrasound-activated nanoemulsions/microbubbles. J Control Release 138:268–276
105. Herman GT (2009) Fundamentals of computerized tomography: image reconstruction from projection, 2nd edn. Springer, Berlin
106. Osborne E, Sutherland C, Scholl A, Rowntree L (1923) Roentgenography of urinary tract during excretion of sodium iodide. J Am Med Assoc 80:368–373
107. Bourin M, Jolliet P, Ballereau F (1997) An overview of the clinical pharmacokinetics of x-ray contrast media. Clin Pharmacokinet 32:180–193
108. Hallouard F, Anton N, Choquet P, Constantinesco A, Vandamme T (2010) Iodinated blood pool contrast media for preclinical X-ray imaging applications—a review. Biomaterials 31:6249–6268
109. Henning T, Weber AW, Bauer JS, Meier R, Carlsen JM, Sutton EJ, Prevrhal S, Ziegler SI, Feussner H, Daldrup-Link HE, Rummeny EJ (2008) Imaging characteristics of DHOG, a hepatobiliary contrast agent for preclinical microCT in mice. Acad Radiol 15:342–349
110. Willekens I, Lahoutte T, Buls N, Vanhove C, Deklerck R, Bossuyt A, de Mey J (2009) Time-course of contrast enhancement in spleen and liver with Exia 160, Fenestra LC, and VC. Mol Imaging Biol 11:128–135
111. Weichert JP, Lee FT Jr, Chosy SG, Longino MA, Kuhlman JE, Heisey DM, Leverson GE (2000) Combined hepatocyte-selective and blood-pool contrast agents for the CT detection of experimental liver tumors in rabbits. Radiology 216:865–867
112. Hallouard F, Briançon S, Anton N, Li X, Vandamme T, Fessi H (2013) Iodinated nanoemulsions as contrast agents for preclinical X-ray imaging: impact of the free surfactants on the pharmacokinetics. Eur J Pharm Biopharm 83:54–62
113. Li X, Anton N, Zuber G, Zhao M, Messaddeq N, Hallouard F, Fessi H, Vandamme TF (2013) Iodinated α-tocopherol nano-emulsions as non-toxic contrast agents for preclinical X-ray imaging. Biomaterials 34:481–491

Chapter 15
Multifunctional Polymeric Nano-Carriers in Targeted Drug Delivery

Ashish Kumar Agrawal, Dileep Urimi, and Sanyog Jain

Abbreviations

ADPA	Anthracene-9, 10-dipropionic acid, disodium salt
ASGP	Asialoglycoprotein
BBB	Blood–brain barrier
BMP	Bone morphogenetic protein
CDs	Cyclodextrins
CH	Chitosan
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
COX	Cyclo oxygenase
DNA	Deoxy nucleic acid
DOX	Doxorubicin
EC	Ethyl cellulose
ECMs	Extracellular matrices
EGF	Epidermal growth factor
EGFP	Expressing green fluorescent protein
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention effect
FA	Folic acid
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FTIC	Fluorescein isothiocyanate

A.K. Agrawal • D. Urimi • S. Jain (✉)
Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics,
National Institute of Pharmaceutical Education and Research (NIPER),
Sector 67, S.A.S. Nagar, Mohali, Punjab 160062, India
e-mail: sanyogjain@niper.ac.in

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_15

GA	Glycyrrhizinic acid
GL	Glycyrrhizin
HA	Hyaluronan
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HER2	Human epidermal growth factor receptor 2
HPMC	Hydroxypropylmethylcellulose
mAb	Monoclonal antibodies
MC	Methyl cellulose
MDR	Multidrug resistance
MEND	Multifunctional envelope-type nano-device
MMPNs	multifunctional magneto-polymeric nanohybrids
MPAP	Myristoylated polyarginine peptides
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
NiMOS	Nanoparticles-in-microsphere oral system
NIRF	Near-infrared fluorescent dye
NPs	Nanoparticles
PAA	Polyacrylic cid
PBLA	Poly (β-benzyl L-aspartate)
PCL	Polycaprolactone
pDNA	Plasmid deoxyribonucleic acid
PEG	Polyethylene glycol
PEI	Polyethylenimine
P-gp	P-glycoprotein
PLA	Poly lactic acid
PLGA	(poly(lactic-co-glycolic) acid)
PLL	Poly (*l*-lysine)
PS	Polystyrene
PSS	Poly(styrenesulfonate)
PTX	Paclitaxel
QD	Quantum dots
RBITC	Rhodamine B isothiocyanate
RES	Reticuloendothelial system
RGD	Arginine–glycine–aspartic acid
RNA	Ribo nucleic acid
scAbPSCA	Single chain prostate stem cell antigen antibodies
scFv	Single-chain fragment variables
siRNAs	Small interfering RNA
SPECT	Single-photon emission computed tomography
SPIONs	Superparamagnetic iron oxide nanoparticles
TfRs	Transferrin receptors
TPGS	D-α-tocopheryl polyethylene glycol succinate

VEGF Vascular endothelial growth factor
WGA Wheat germ agglutinin
γ-PGA γ-polyglutamic acid

15.1 Introduction

"Going small to achieve big wonders" is the motto of nanotechnology. Recent advances in nanotechnology based drug delivery are opening wide horizons for the targeted delivery of therapeutics. For targeted delivery, it is of immense importance to fabricate delivery vehicles which can deliver the drug exclusively at the targeted site with minimal effect on the healthy tissues [1]. Moreover, there is a strong need to develop disease specific delivery vehicles which not only deliver the drug at the disease site but can also serve additional functions such as tissue imaging. This beacons for the designing of multifunctional nanoparticles (NPs) which can perform in a smarter way by providing targeting, therapy and imaging "all in one" system (Fig. 15.1). This "all in one" strategy represents a multimodal approach for the battle against grave diseases like cancer, autoimmune disorders, diabetes, etc.

With the advent of era of nanotechnology in drug delivery, numerous nano-carriers such as liposomes, micelles, dendrimers, polymeric NPs, solid lipid NPs and metallic NPs have been employed to find solutions for incurable diseases [2–8]. Next generation of nanomedicine deals with the fabrication of multifunctional nano-scopic vectors capable of ferrying combinations of drugs, targeting ligand and image contrast agents to the disease site in the same vehicle. Among the delivery platforms exploited for multifunctionality, polymeric NPs or self-assembled micelles from amphiphilic block copolymers are particularly of interest to the drug delivery scientists owing to the flexibility of attaching numerous targeting ligands to achieve the site-specific delivery or modulation of polymeric backbone to alter the release rate of drugs.

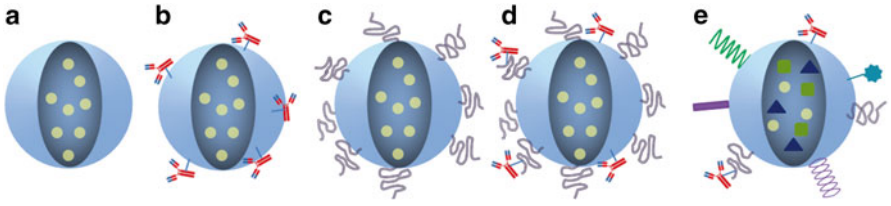

Fig. 15.1 Evolution of multifunctionality: foundation to current scenario a. Stereotyped NPs with drug entrapped inside ( ) b. Polymeric NPs targeted with covalently bound antibody ( ) c. Stealth NPs surface modified with PEG ( ) d. Polymeric NPs with both stealth and immunogenic nature by attaching PEG and antibody individually to surface of nanoparticle or attaching the antibody to PEG chain which is already attached with nanoparticle e. Advanced NPs with multifunctionality in terms of surface attachment of PEG or PEG combined with antibody for targeting and immunogenicity; attachment of diagnostic tools ( ); attachment of cell penetrating peptide ( ); attachment of viral components ( ); attachment of stimuli sensitive materials ( ); entrapment of gold/silver materials ( ) or magnetic NPs ( ) for live imaging

Polymeric NPs are primarily composed of natural or synthetic polymers which can be modulated in a variety of ways to achieve particles with desirable characteristics. Most commonly employed biodegradable polymers include poly (D,L-LACTIDE-CO-glycolide), poly(ε-caprolactone), poly(β-amino esters), chitosan, hyaluronic acid, dextran, etc. [9]. The primary advantages of using polymers in the formulation of nanoparticulate drug delivery vehicles are: (1) Wide range of polymers to choose polymer of interest which is biodegradable in nature (2) They offer lots of sites for further functionalization and attachment of targeting ligands to achieve site-specific drug delivery (3) These polymeric NPs offer flexibility of incorporation of other metallic NPs decorating the composite particles with optical, magnetic or hyperthermic characteristics [10]. These variant properties strive to achieve true multifunctionality in the drug delivery vectors where drug targeting, delivery and tissue imaging can be accomplished all in one go. Although the concept of multifunctionality has shown tremendous application in the field of drug delivery cum therapy, there are some major challenges which need to be overcome if successful translation from bench-top research to bedside is required to be achieved for these systems.

15.2 Polymers

A variety of natural, semisynthetic, and synthetic polymers have been exploited in the fabrication of multifunctional polymeric nano-carriers (Fig. 15.2).

Advances in the polymer technology resulted in the emergence of wide range of polymers and among them; biodegradable polymers attracted the considerable attention

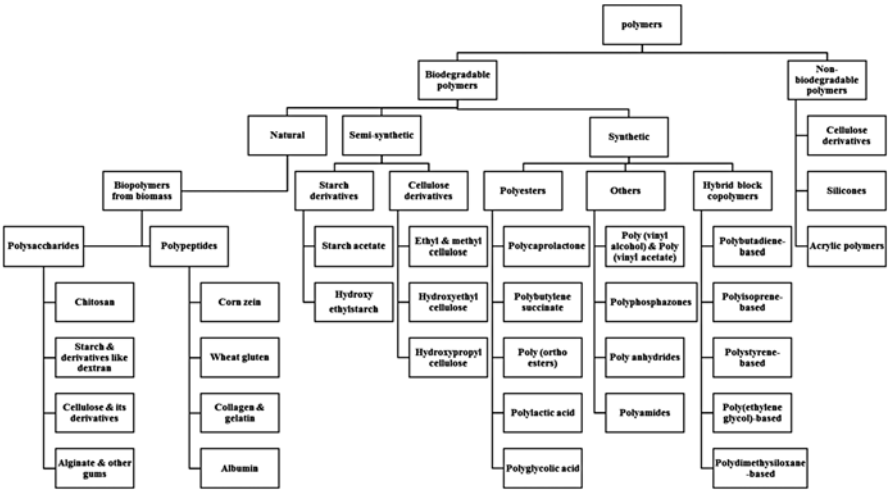

Fig. 15.2 Classification of polymers

and extensively investigated in the field of drug delivery. Moreover, inherent character of providing multiple attachment sites for further functionalization with targeting ligands to achieve site specific drug delivery further revolutionize the field. As present chapter deals with the delivery application of multifunctional nano-carriers, only some extensively studied biodegradable polymers are covered in this section.

15.2.1 Natural Polymers

Human being has been blessed by a number of gifts of Mother Nature and natural polymers with diverse characteristics are one of them. Although these polymers are equipped with lots of inherent characteristics, biodegradability and nontoxicity are two major factors which make these polymers best suitable for drug delivery purpose.

15.2.1.1 Cellulose

Cellulose is a water insoluble polysaccharide with the formula $C_6H_{10}O_5$ consists of D-glucose units linked by β 1,4-glycosidic bond and constitute the major component of cell wall of many organism. Due to the insolubility, it has not been used frequently in the field of drug delivery [11].

15.2.1.2 Alginates

Alginate is a naturally occurring, anionic, linear copolymer of (1→4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues and the final characteristics of the polymer depends upon the G:M ratio in final polymer. This is a low-cost polymer and its structural resemblance with living tissues make it suitable for different biomedical application [12].

15.2.1.3 Dextran

It is a natural, colloidal, biocompatible, and nontoxic polymer which is highly resistant to protein adsorption [13]. Dextran, alone or in combination, has been reported for the fabrication of delivery vehicles for targeted drug delivery.

15.2.1.4 Albumin

Albumins are a family of globular proteins which broadly belongs to the class of polypeptides. Bovine serum albumin (BSA) and Human serum albumin (HSA) are the major albumins which are well reported as well established vehicle for the delivery of bio-actives [14].

15.2.1.5 Gelatin

Gelatin is derived from collagen, a natural protein, which occurs in the skins, bones, and connective tissues of animals and belongs to the class of polypeptides. Gelatin is translucent, colorless water insoluble polymer which is made soluble by hydrolysis and extensively studied in the field of drug delivery [9].

15.2.2 Semisynthetic Polymers

15.2.2.1 Chitosan

Chitosan is a biocompatible, biodegradable, and nontoxic polymer which has studied extensively in the field of drug delivery. Ease of surface modification, mucoadhesion, penetration enhancement, are some of the positive attributes which make the chitosan the most favorable and extensively studied polymer for drug delivery. However, solubility in acidic media and poor stability in physiological conditions are the associated drawback. A variety of chitosan derivatives have been developed to overcome these limitations [15, 16].

15.2.2.2 Cellulose Derivatives

Among the different semisynthetic polymers, cellulose derivatives like methyl cellulose (MC), ethyl cellulose (EC), hydroxyl propyl cellulose (HPC), and hydroxypropylmethylcellulose (HPMC) have been used extensively for different biomedical applications [17, 18].

15.2.3 Synthetic Polymers

15.2.3.1 Polyesters

Although a variety of hydrolytically labile polyesters have been evaluated for biomedical application, poly(glycolide), poly(lactide), copolymers of poly(lactide-co-glycolide) and poly caprolactone are the universal choice due to their safety and flexibility to be easily managed into a variety of delivery vehicles.

Polylactide (PLA)

It is biodegradable and thermoplastic aliphatic polyester obtained from various natural sources of starch, viz., corn and sugarcane. The lactide monomer possesses two chiral carbons, and hence the properties of the polymer depends upon the monomer

units, i.e., D-lactide (the D-,D- cyclic dimer), L-lactide (the L-,L- cyclic dimer), or the meso-lactide (the D-,L- cyclic dimer). However, PLA is most commonly synthesized by copolymerization of a racemic mixture of the D- and L-lactide monomers [19].

Polyglycolide (PGA)

It is a synthetic biodegradable and highly crystalline polymer which is insoluble in most of the organic solvents because of its high crystallinity. This polymer is very sensitive towards hydrolytic degradation and produces degradation products which are acidic in nature which limit this polymer in tissue engineering applications [20].

Poly (Lactide-co-Glycolide) (PLGA)

It is a biocompatible, biodegradable, FDA approved copolymer of polylactic acid and polyglycolic acid. PLGA is nontoxic as hydrolysis results in the production of lactic acid and glycolic acid which are the byproducts of several metabolic pathways. However, relatively higher cost is the drawback of this polymer [20].

Polycaprolactone

It is a biodegradable, FDA approved polymer for drug delivery device and suture formation. High production cost is the drawback; however, combination with other natural and biodegradable polymers makes it cost-effective and also enhances the flexibility and biodegradability [21].

15.2.3.2 Polyamides

γ-Polyglutamic Acid

It is a naturally occurring anionic polymer which is obtained from some of the Bacillus species and made of L-glutamic acid residues connected by amide linkages. This is biodegradable, nontoxic, and water soluble polymer which has been investigated extensively in fabricating nano-carriers for targeted drug delivery [22].

15.3 Levels of Multifunctionality in Polymeric NPs

The levels of multifunctionality can be broadly categorized in the following two categories:

15.3.1 First Level of Multifunctionality

This includes simple modification of the surface of polymeric nano-carriers with the targeting ligands for active targeting or "PEGylation" of their surface so as to prolong the blood circulation time and avoidance of uptake by reticuloendothelial system (RES). Multiple loading of drugs within the same vesicle to achieve combinative therapeutic effect particularly in case of cancer chemotherapy can also be considered under the first level of multifunctionality.

15.3.2 Second Level of Multifunctionality

This includes complex multifunctionality of the polymeric nanoparticles such as combining targeting, imaging, and therapy in one system. It involves the use of nanoparticles beyond its role as mere vectors to deliver the drug or imaging agent.

15.4 Multifunctional Polymeric Nanoparticles for Targeted Cancer Therapy

For the last many decades chemotherapy and radiation therapy have been immerged as major options to treat a wide variety of cancers. Although lots of newer chemotherapeutic agents and advancement have come into picture, the cancer therapy is quite far from what is desired. The efficacy of chemotherapy majorly depends on the ability of the therapeutics to selectively attack on the tumor site while minimally affecting the healthy cells. To achieve this objective, decades of research have been focused to develop such smart delivery vehicles which can preferentially localize the chemotherapeutic agents to the tumor site. As a result of extensive research nano-carriers based technologies came into the picture which further revolutionized the field of cancer therapy. Rapid growth of solid tumors represent fenestrated vasculature and poor lymphatic drainage, resulting in enhanced permeability and retention (EPR) effect [23], which permits nano-carriers to accumulate preferentially at the tumor site (Fig. 15.3).

Hence, the preferential accumulation of the NPs at tumor site demonstrate improved efficacy by simultaneously mitigating the nonspecific side effects of chemotherapeutics due to undesired distribution to the normal tissues. Although chemotherapeutic agents entrapped within the NPs are protected from the metabolism and rapid clearance, PEGylation provides the stealth shielding and avoids rapid uptake by macrophages and mononuclear phagocytes of the reticuloendothelial system (RES) [24, 25]. Besides this inherent passive targeting property, the tumor localization can further be improved by active targeting in which nano-carriers can be decorated with number of small molecules like folic acid [26], thiamine [27] and even antibodies or lectins [28] which are preferentially recognized by tumor cells. This

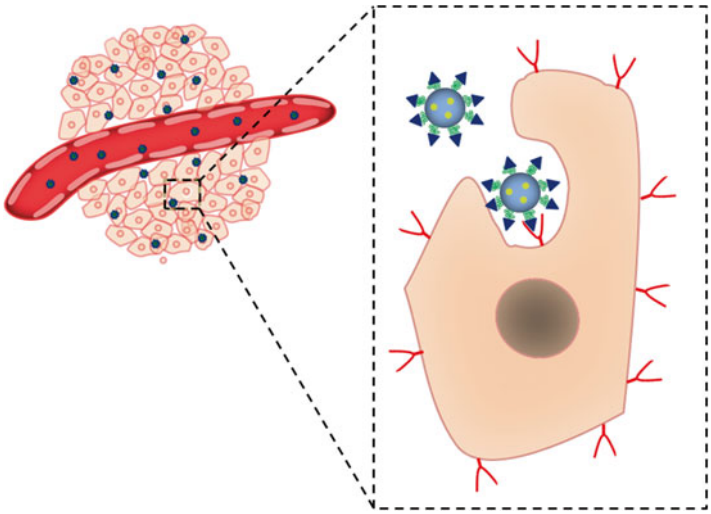

Fig. 15.3 Mechanism of drug accumulation in cancerous tissue through multifunctional nanoparticles

platform could further be explored for imaging, diagnostics and guided therapy by combining the imaging/diagnostic agents within a system. Therefore, these nano-carriers have been made smarter and multi-functional by combining the tumor targeting, tumor therapy and tumor imaging in a single system which presents an effective alternative against cancer. A variety of biocompatible and biodegradable polymers have also been used to prepare nano-carriers for tumor-targeted delivery viz. poly(D,L-lactide-co-glycolide), poly(ε-caprolactone), and poly(β-amino esters) [29–32].

15.4.1 Multifunctionality by Targeting

In one of the approach to make the nano-carriers multifunctional, tumor specific targeting moieties are appended on the NPs surface which further directs the trafficking of NPs following administration without affecting the physical, chemical, and biological properties of the carrier system or chemotherapeutics entrapped within the delivery vehicle. This targeting strategy will provide the additional benefits of targeting all types of solid tumors as well as metastatic cells and cancerous leukocytes. These targeting moieties can be broadly classified as receptor ligands (peptides, vitamins, and carbohydrates), proteins (antibodies and their fragments), and nucleic acids (aptamers). Interaction of growth factor or vitamins with cancer cells represents a commonly used targeting strategy, as receptor for these moieties are overexpressed over the cancer cells to fulfil the need of fast-growing metabolism. Epidermal growth factor (EGF) has been shown to interact with EGF receptor, overexpressed in breast and tongue cancer [33]. Similarly, vitamin folic acid (folate) has also been reported to interact with folate receptors overexpressed in a variety of

Table 15.1 Different types of receptors which are overexpressed on different tumors

S. no	Ligand	Receptor	Type of tumor	Reference
1.	Transforming growth factor-α (TGF-α)	Epidermal growth factor receptor (EGFR)	Non-small-cell lung cancer (NSCLC)	[36]
2.	Epidermal growth factor (EGF)	EGFR	Human pancreatic cancer	[37]
3.	Platelet-derived growth factor (PDGF-A)	Platelet-derived growth factor receptor α (PDGFR-α)	Breast cancer	[38]
4.	Heregulin (HRG)	ErbB3 or ErbB4	Breast and ovarian cancer	[39]
5.	Folic acid	Folate receptors (FRs)	Ovarian, endometrial, and kidney cancer	[34]
6.	Transferrin (Tf)	Tf receptors (TfRs)	Pancreatic, colon, lung, and bladder cancer	[35]
7.	Thrombin	Protease-activated receptors 1(PAR1)	Breast, colon, head and neck, prostate cancer	[40]
8.	Gastrin releasing peptide (GRP)	Gastrin-releasing peptide receptors (GRPR)	Small-cell lung and pancreatic cancer	[41, 42]
9.	Luteinizing hormone	Luteinizing hormone receptors	Testicular cancer	[43]
10.	Thyroid stimulating hormone	Thyroid stimulating hormone receptors	Thyroid cancer	[44]

tumor cells such as ovarian, endometrial, and kidney cancer [34]. Transferrin receptors (TfRs) have also been reported to be overexpressed over a variety of tumor cells including pancreatic, colon, lung, and bladder cancer) and conjugation of transferrin with NPs has been reported as effective strategy to target the cancer cells [35]. Table 15.1 summarizes the different receptors which are overexpressed on different type of tumors and can be a viable option for designing the delivery vehicle which selectively deliver the drug to the tumor site.

Nano-carriers ornamented with targeting ligands have been resulted in improved outcome in different animal tumor models [45–47]. The expression of folate and Tf is correlated with the metabolic rate and these receptors are also overexpressed in fast growing healthy cells such as fibroblasts, epithelial and endothelial cells. Targeting with these moieties to cancer cells may lead to nonspecific targeting and enhanced toxicity [48]. Arginine–glycine–aspartic acid (RGD), ligand of the cell adhesion integrin αvβ3 on endothelial cells, has been reported to improve intracellular drug delivery in different murine tumor models [49, 50]. However, nonspecific interaction of RGD with other integrins such as α5β1 and α4β1 may result in toxicity to normal cells. In addition, heparin sulphate, chondroitin sulphate, and hyaluronic acid (HA), have also been recognized as effective targets for specific extracellular matrices (ECMs) receptors [51, 52]. Upadhyay et al. fabricated

biomimetic DOX loaded Polymersomes from Hyaluronan-block-Poly(γ-benzyl glutamate) copolymers (PolyDOX). The polymer were synthesized using click chemistry principles, yielding nano-vesicles with excellent colloidal stability, controlled size, and capability to encapsulate both the hydrophilic as well as hydrophobic drugs [53]. Intracellular delivery PolyDOX in high (MCF-7) and low (U87) CD44 expressing cancer cell models was further extensively studied. DOX was successfully encapsulated via nanoprecipitation. PolyDOX exhibited enhanced uptake by MCF-7 than U87 cells, as analysed by flow cytometry. Microscopy imaging revealed that in MCF-7 cells PolyDOX was more in cytoplasm and free DOX in nuclei, whereas in U87 cells free DOX was also found in the cytoplasm. Furthermore, PolyDOX showed greater suppression of breast tumor in female Sprague–Dawley (SD) rats as compared to phosphate buffer saline pH 7.4 (PBS) control group. Loading of DOX within polymersomes further reduced the cardiotoxicity of DOX. These results were conclusive to suggest that intracellular delivery of PolyDOX was dependent on the CD44 expression level in cells due to presence of hyaluronic acid on the surface of polymersomes [54]. In yet another study carried by Upadhyay et al., DOX-loaded poly(γ-benzyl *l*-glutamate)-block-hyaluronan (PBLG23-b-HYA10)-based polymersomes (PolyDOX) were prepared and efficiently labelled with technetium-99m radionuclide for in vivo studies. PolyDOX exhibited enhanced blood circulation time. Bio-distribution studies revealed selective accumulation of DOX loaded polymersomes in the Ehrlich Ascites Tumor (EAT) as a result of passive accumulation and active targeting (CD44-mediated endocytosis) in EAT-bearing mice. The life span of PolyDOX treated mice group was increased nearly six times compared to DOX treated group and PolyDOX markedly controlled tumor growth. Toxicity studies conducted further established the safety of PolyDOX as no hemolysis was observed at equivalent dose of 200 μg/ml of DOX [55, 56]. Pang et al. developed brain drug delivery system for glioma chemotherapy based on transferrin-conjugated biodegradable polymersomes, Tf-PO-DOX. Nanoprecipitation methodology was utilized to prepare DOX containing polymersomes, which were then conjugated to transferrin to form vesicles of 107 nm size. On an average, 35 molecules of transferrin were found to be present on the surface of each polymersome. Pharmacokinetics and brain distribution studies further revealed the significant accumulation of transferrin conjugated polymersomes in the brain. Pharmacodynamic studies revealed marked reduction in tumor volume and significant increase in median survival time in the group treated with Tf-PO-DOX in comparison with control saline treated animals and animals treated with PO-DOX and free DOX solution [57].

In yet another strategy of targeting, monoclonal antibodies (mAb) was first described by Milstein in 1981 and afterwards this approach demonstrated clinical efficacy and later different mAb were approved by US Food and Drug Administration (FDA) [58, 59]. mAb rituximab (Rituxan), Trastuzumab (Herceptin, an anti-HER2 mAb), Bevacizumab (Avastin, an anti-VEGF mAb) were approved for non-Hodgkin's lymphoma, breast cancer, and colorectal cancer, respectively [60, 61]. The approach was so successful that many of the delivery systems are in preclinical and clinical trials [62, 63]. Furthermore, advancement in the antibody engineering

resulted in the generation of antibodies of animal and human origin such as chimeric mAbs, humanized mAbs, and antibody fragments. Antibodies may be used in their innate state or as fragments; however, use of whole mAbs is advantageous over the fragments as it proposes two binding sites which further results in higher binding efficiency and long term storage. Although antibody fragments such as antigen-binding fragments (Fab), dimers of antigen-binding fragments (F(ab′)2), single-chain fragment variables (scFv) are less stable than whole antibodies, they are quite safer owing to reduced nonspecific binding [62, 63]. Furthermore, the efficacy of antibodies can be improved by conjugating the mAb with therapeutic agents. Conjugation of anti-CD33 antibody with calicheamicin was the first clinically approved formulation for targeting cancerous cells. Although the efficacy of immunoconjugates is clinically approved, lethal side effect due to nonspecific binding is still a challenge [64]. Although BR96-DOX immunoconjugate that targets and binds to the Lewis-Y antigen (overexpressed on breast cancers) demonstrated remarkably higher antitumor activity and lower toxicity, severe gastrointestinal toxicity due to binding of the conjugate to Lewis-Y-related antigens expressed by non-targeted gastrointestinal epithelial cells was also reported [65, 66]. Aptamers, which are short single-stranded DNA or RNA oligonucleotides and selectively, bind to a wide variety of targets (intracellular and transcellular proteins), have also been developed to selectively bind to specific receptors on cancer cells [67]. Docetaxel loaded and aptamer decorated NPs that specifically target the antigen on the surface of prostate cancer cells, revealed high selectivity and efficacy [33]. In another approach to develop targeted delivery for the treatment of cancer, Debets et al. utilized a single-domain antibody (A12) that specifically targets PlexinD1 (a transmembrane protein overexpressed in tumor vasculature) and grafted it on the surface BCN-functionalized polymersomes using a strain-promoted azide alkyne cycloaddition. In vitro studies revealed the potential of these polymersomes to target tumor-vessel VHH A12 [68].

15.4.2 Multi-Functionality by Combination of Multiple Therapeutics

In the early era of research, towards the cancer treatment, different delivery systems were designed to improve the efficacy of already reported therapeutics by focusing on the solubility and permeability related issues related to individual chemotherapeutic. Although this approach was quite effective in comparison to the conventional delivery vehicles yet was not up to the mark due to the complexity of cancer biology. By considering the complex biology of different cancers, combination approaches came into picture in which multiple of therapeutics were co-encapsulated in a single delivery system to target the cancer at multiple levels. Multifunctional nano-carriers loaded with combination of therapeutics offer an effective platform for effective cancer therapy.

15.4.2.1 Antiangiogenic and Cytotoxic Drug Combination

Angiogenesis has been recognized as organizing principle of biology in which new blood vessels are generated for development, reproduction and repair of the biological system. The rate of angiogenesis is certainly higher in certain cases like cancer, which is required to fulfil the higher demands of nutrition for growth and metastasis of fast proliferating cancer cells and inhibiting vessel formation by antiangiogenic agents offers hope for effective treatment of cancer. A wide range of factors including, but not limited to, fibroblast growth factor (FGF), insulin-like growth factor, G-CSF, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), have been recognized for angiogenesis and targeting to these factors can be exploited as a potential target for cancer therapy [69, 70]. However, administration of antiangiogenic alone produced only modest response in clinical trials [71, 72] and did not found much effective for long-standing survival welfares [73]. Later it was found that combination of these antiangiogenic drugs with cytotoxic agents can fortify the response to a greater extent. This combinatorial concept was proved in many reports; paclitaxel and DOX in combination with JNJ17029259 (VEGF receptor 2 inhibitor) [74]; cisplatin and gemcitabine in combination with bevacizumab (anti-VEGF) [75]; temozolomide in combination with COX-2 inhibitors, which indirectly inhibit VEGF function [76]. In clinical settings too, chemotherapeutics (irinotecan, fluorouracil, and leucovorin) in combination with bevacizumab, an antibody targeted against the VEGF, produced an exceptional increase in survival (up to 5 months) in colorectal cancer patients [77–79]. This combinatorial treatment strategy can be made more effective by modulating the release pattern of both the co-encapsulated bio-actives. On the similar line, a multifunctional nanoparticulate formulation was reported in which the release of both the agents was designed in such a way that after accumulating the NPs at tumor site, antiangiogenic drug (combretastatin A4) first releases which suppress the tumor vasculature and then exhibit sustained release of the cytotoxic agent DOX [80]. The positive findings of the above report clearly suggest that, the multi-functional nano-carriers that work on multiple levels can be exploited as promising delivery vehicle for fortified antitumor efficacy.

15.4.2.2 Multidrug Resistance Modulators with Chemotherapeutics

Multidrug resistance (MDR) can be defined as the resistance of cancer cells to a diverse range of structurally and functionally unrelated drugs and is considered as principal mechanism by which resistance to chemotherapeutics is developed by many cancers and considered as one of the major reason for chemotherapy failure. MDR can be classified as intrinsic (inherent resistance of cancer cells against chemotherapy) and acquired (tumor reversions after treatment) [81]. To overcome this barrier of chemotherapy a variety of drugs were recognized which check the phenomena of cancer cells to become resistance against chemotherapy. Multiple mechanisms for MDR development have been recognized which include adaptation

of additional mechanism for DNA repair, enhanced production of drug metabolizing enzymes leading to the inactivation of chemotherapeutics, blockade of the apoptotic signalling cascade which leads to the enhanced survival of cancerous cells, and activation of efflux pump such as P-glycoprotein (P-gp) for pumping the already absorbed drug out from the cell [82–87]. Among the different strategies, activation of efflux pump has been recognized as the most prominent for developing the MDR resistance in cancer cells. To target this particular lacuna, different first generation P-gp inhibitors like verapamil, cyclosporine A [88] came into picture; however, systemic administration of these agents to achieve the desired therapeutic efficacy resulted in undesired toxic effects [89]. In the course of the research, dexverapamil or dexniguldipine, glibenclamide, tariquidar, PSC833, GF120918, XR9576, LY335979 were developed with more specificity and high affinity at nanomolar concentration and lesser side effects [90, 91]. Administration of these agents along with chemotherapeutics in nano-carriers demonstrated improved efficacy and reduced toxicity which could be attributed to the inherent tendency of nano-carriers to be up taken by endocytosis or phagocytosis leading to the accumulation at the centre of the cell which is quite far from the efflux pump [92]. Accumulation of these nano-carriers at the centre of the cell also provide the drug release at much higher rate in comparison with the efflux rate which further overcome this barrier and maintain the therapeutic level of chemotherapeutics for a longer period of time. A variety of nanoparticulate systems, polyalkylcyanoacrylate NPs having the combination of DOX and cyclosporin A [93], poly(D,L-lactide-co-glycolide) NPs for the delivery of paclitaxel and tariquidar [94] have been reported for successful delivery of these drugs. Positive outcomes of many of the clinical studies [95, 96] further support the suitability of this combination strategy for cancer therapy.

15.4.3 Complex Multi-functionality

Although lots of newer formulation approaches have been developed for the delivery of chemotherapeutics more accurately and specifically to the tumor site, the therapy has not yet performed up to the mark which could be attributed to the lack of advanced diagnostic approaches. Usually, the cancer is detected at an advanced stage, when the primary tumor has been metastasized and invaded to other organs. Current approaches like chemotherapy and radiation therapy have relatively poor specificity toward malignant tissues hence the disease is still not curable. Therefore, to fortify the cancer prevention and therapy novel probes for diagnosis and novel ferrying cargo must be fabricated which can diagnose the disease at early and curable stage and deliver the drugs selectively and effectively to the cancerous cells/tissues without causing collateral damage to the normal tissues. With the advent in the field of chemotherapy, smarter multifunctional nano-carriers came into limelight in which core imaging agents such as iron oxide, gold, gadolinium and quantum dots were also incorporated along with chemotherapeutics to have the advantages of alternate anticancer therapies like hyperthermia, radiation and photodynamic therapy (Table 15.2). In line with the concept, many reports focused on

Table 15.2 Different combinatorial treatment strategies used to treat cancer by using multifunctional polymeric nano-carriers as delivery platform

Entrapped moiety	Nanotechnology platform	Multifunctionality	Reference
Drug therapy for synergistic efficacy			
Paclitaxel and C6-ceramide	PLGA/PbAE (70:30 by weight) NPs	Synergistic efficacy by using apoptotic signalling molecule along with chemotherapeutics for MDR cancer treatment	[109]
DOX and gold	Poly(lactic-co-glycolic acid)-gold half-shell NPs (PLGA H-S NPs)	Synergistic efficacy by using heating along with chemotherapeutics	[110]
Drug therapy with targeting potential			
Paclitaxel	PLGA NPs	Surface functionalization with A10 RNA aptamer for prostate cancer targeting	[111]
Paclitaxel	Poly(D,L-lactide-co-glycolide)/ montmorillonite NPs	Functionalisation with mAB (Trastuzumab) for targeting breast cancer	[112]
Docetaxel	Poly(lactide)-TPGS NPs	Functionalization with Trastuzumab for targeting breast cancer	[113]
Paclitaxel	PLGA NPs	Functionalization with RGD peptide to target Tumor endothelium	[114]
Paclitaxel	PLA-PEG NPs	Surface functionalization with biotin and folic acid	[115]
Drug therapy with imaging			
Fe_3O_4	Fe_3O_4 NPs loaded polystyrene nanospheres (PS NSs)	Surface functionalization with QDs to use the hyperthermia along with fluorescent imaging	[116]
Paclitaxel	Chitosan NPs	Co-encapsulation of drug with Cy5.5 for early stage detection and therapy	[108]
Paclitaxel	PLGA NPs	Co-encapsulation of drug with SPIO for drug delivery and real time imaging	[117]
Therapy with targeting and imaging			
Photofrin	PAA NPs	Surface functionalization with PEG and RGD along with co-encapsulation of MRI contrast agent for live imaging	[118]
Photofrin	Polyacrylamide NPs	Functionalization with F3 peptide and co-encapsulation with SPIO for enhanced efficacy	[119]

(continued)

Table 15.2 (continued)

Entrapped moiety	Nanotechnology platform	Multifunctionality	Reference
CCL21	Chitosan NPs	Surface functionalization with folic acid along with co-encapsulation of quantum dots	[120]
DOX	Polymeric NPs	Surface functionalization with anti HER antibody along with magnetic nanocrystals as contrast agent	[106]
DOX	PLGA NPs	Functionalization with folic acid along with magnetite and QDs for optical imaging	[107]
Docetaxel	PLGA NPs	Functionalization with folic acid along with QDs for optical imaging	[121]
DOX	Heterobifunctional amphiphilic triblock copolymers R (R = methoxy or folate (FA))-PEG_{114}-PLA_x-PEG_{46}-acrylate)	Surface functionalization with folic acid along with SPIO as MRI contrast agent	[122]
DOX	Poly(ethylene oxide)-trimellitic anhydride chloride-folate (PEO-TMA-FA) NPs	Surface functionalization with folic acid along with SPIO as MRI contrast agent	[123]
Docetaxel	PLGA NPs	Functionalization with single chain prostate stem cell antigen antibodies (scAbPSCA) with SPIO for targeting along with imaging	[124]
Drug therapy with photothermal and imaging			
Paclitaxel	PLGA NPs	Functionalization with poly(styrenesulfonate) (PSS)-coated Au nanorods with QD/ Fe_3O_4	[125]
Paclitaxel	Polystyrene and PLGA based NPs	Functionalization with Fe_3O_4 loaded PSS matrix and QDs	[126]
Cisplatin	Chitosan NPs	Functionalization with gold nanorods for phototherapy as well as imaging	[127]

multifunctional nanomedicines for multimodal imaging [97–101] and simultaneous diagnosis and therapy [102–104] were published. Muthiah et al. reported surface tuneable polymersomes decorated with different polymers like positively charged polyethylenimine (PEI-P), neutrally charged polyethylene glycol (PEG-P), and negatively charged glycine (GLY-P) were attached to polymer backbone polysuccinimide (P). These polymersomes were then loaded with super paramagnetic iron oxide nanoparticles and anticancer drug paclitaxel for image guide chemotherapy. PEG modified polymersomes showed enhanced retention in liver and spleen in in vivo hemi-spleen mouse metastatic liver model. Pourtau et al. developed antibody grafted maghemite loaded polymersomes as novel MRI contrast agents for bone metastasis imaging. Bone tumor was developed in mice using human breast cancer cells since in 80 % cases, breast cancer preferentially metastasizes to the skeleton, e.g., femur, pelvis, vertebrae, and skull. In vivo MRI imaging demonstrated targeting and enhanced retention of antibody labelled polymersomes at the tumor site in animal model [105].

Nasongkla et al. developed the multifunctional polymeric micelles capable of simultaneous tumor imaging and delivery of anticancer agents. DOX and a cluster of superparamagnetic iron oxide NPs (SPIONs) were successfully embedded inside the micelle core and the targeting ligand cRGD was conjugated on the micellar surface. This enabled these multifunctional NPs to be targeted to avβ3-expressing cancer cells. In vitro MRI and cell cytotoxicity studies demonstrated ultrasensitive MRI imaging and avβ3-specific cytotoxic response of these multifunctional NPs. These systems served as a single platform which can be used to detect tumors, treat them, monitor treatment response and guide therapeutic regimens [104]. In line with the idea of multifunctionality, Yang et al. reported multifunctional magneto-polymeric nanohybrids (MMPNs) comprising magnetic nanocrystals as ultrasensitive MR contrast agents, anticancer drugs as chemotherapeutic agents, anti-HER antibody as targeting ligand and biodegradable amphiphilic block copolymers as stabilizers. To obtain the required water solubility, the hydrophobic nanocrystals (MnFe2O4, Fe3O4) and anticancer drug (DOX) were simultaneously encapsulated with the amphiphilic block copolymer by a nanoemulsion method to give MMPNs. To make the system targetable against human epidermal growth factor receptor 2 (HER2), NPs were conjugated with anti-HER antibody (HER, herceptin) by utilizing the carboxyl group on the surface of the MMPNs. In line with the author's hypothesis, antibody-modified MMPNs (HER-MMPNs) demonstrated ultrasensitive targeted detection by MRI in in vitro and in vivo models and exhibited excellent synergistic effects for the inhibition of tumor growth [106]. In one of the interesting work multifunctional polymer nanomedical platform were reported for simultaneous cancer-targeted magnetic resonance (MR) or optical imaging and magnetically guided drug delivery by using biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) as a matrix forming polymer. In the current strategy DOX was used as chemotherapeutic agent along with superparamagnetic magnetite nanocrystals (nanocrystals of Fe3O4 or CdSe/ZnS) and semiconductor NPs (quantum dots) for magnetically guided delivery and T2 MRI contrast agent and optical imaging, respectively. Finally, PLGA NPs were also decorated with folate for targeting the

KB cancer cells. Following the treatment with the multifunctional polymer NPs cancer cells were detectable through MRI or confocal microscopy and magnetic guiding was reported to enhance the cancer-targeting efficiency of the multifunctional polymer nanomedicine [107]. In another set of study theragnostic multifunctional tumor-homing NPs were developed by using chitosan as matrix forming base polymer, paclitaxel (PTX) as chemotherapeutics and Cy5.5, a near-infrared fluorescent (NIRF) dye, for simultaneous execution of cancer diagnosis and therapy (cancer theragnosis). Cy5.5 labeled PTX loaded chitosan NPs exhibited significantly increased tumor-homing ability with low nonspecific uptake by other tissues in SCC7 tumor-bearing mice [108]. Although multifunctional polymeric nano-carriers have shown exciting results in various in vitro and in vivo models, the efficacy needs to be established in clinical settings. Table 15.2 summarizes the different simple and complex multifunctional strategies in nut shell for efficient delivery of different therapeutics as described in Sects. 4.2 and 4.3.

15.5 Multifunctional Polymeric Nano-Carriers for Gene Delivery

Gene therapy has been evolved as a potential therapeutic strategy for various stubborn human diseases like cancer, Parkinson's, Alzheimer's, etc. since the last few decades. Gene therapy refers to the administration of a strand of nucleic acid (DNA, RNA, or a chemical analogue) that specifically bind to the messenger RNA (mRNA) produced by a particular gene causative of a particular disease and turning that gene "off" [128–130]. Therapeutic effectiveness and safety of this therapy depends on the ability of ferrying cargo to deliver the gene construct to its target site while avoiding deleterious consequences to normal cells. Figure 15.4 represents the schematic delivery of genetic material to target site by using the concept of multifunctionality.

Safety risk and immunogenicity associated with the conventional viral vectors resulted in emergence of nonviral vectors as a potential alternative to them [131–133]. Advantages, like ease of preparation, non-immunogenicity, ability to shield the genetic material against nucleases, high loading capacity, feasibility of attaching targeting ligand, and low cost associated with non-viral vectors [134, 135] shifted the focus of scientific community towards this germane area which introduced a number of nonviral vectors, viz., cationic polymers, cationic liposomes, and dendrimers for gene delivery [136, 137]. These cationic vectors electrostatically interact with negatively charged phosphate groups of DNA and condense DNA to submicron size particles facilitating its cellular uptake [138]. Additionally, non-viral vectors can be explored as a common platform in which concept of the genetic material can be combined with therapeutic moiety and/or imaging agents to further improve the performance of the therapy. On the line of combining genetic material with therapeutics HO Kin and coworkers encapsulated Bcl-xL siRNA and DOX

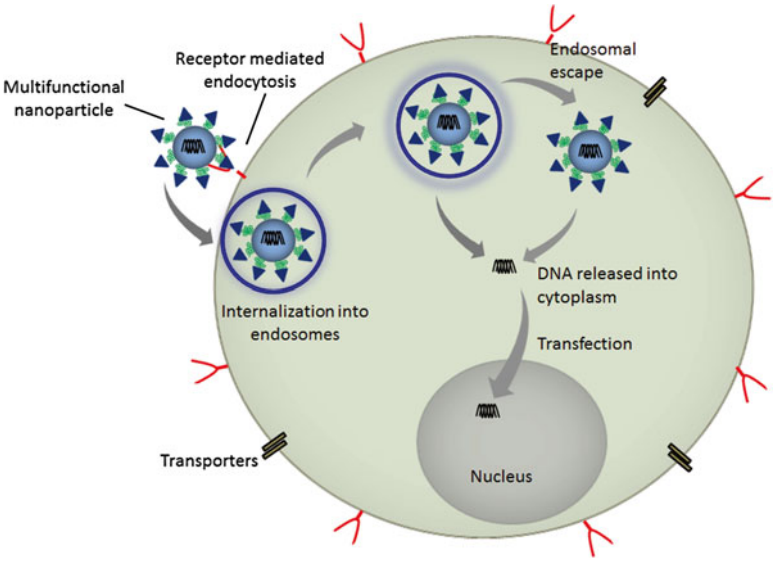

Fig. 15.4 Schematic representation of targeted delivery of genetic material by using multifunctional nano-carriers

into designed methoxy-poly(ethylene glycol)-block-poly(D,L-lactic acid) (mPEG-b-PLA) block copolymer polymersomes. Cytotoxic studies conducted in MKN-45 and MKN-28 human gastric cancer cell lines demonstrated significant cytotoxicity activity at low DOX dose in dual therapy compared to only gene and drug loaded ones. This study demonstrated effective dual therapeutic action of chemotherapy and apoptosis induction via selective gene silencing and activation [139]. Younghoon et al. synthesized non-ionic polymersomes for the delivery of siRNA and antisense oligonucleotides, AON. These degradable particles were passively taken up by cells and capable of escaping endosomes by conversion into micelles in the cellular environment. siRNA was released into cytosol for mRNA breakdown while AON was delivered to the nucleus for exon skipping within pre-mRNA. This knockdown was as effective as cationic lipid transfection and half as effective as Lentivirus. Preliminary studies in mouse model of muscular dystrophy following intramuscular injection of AON loaded polymersomes lead to the expected protein expression along the entire length of muscle [140]. Nano-vector engineering offers great promise in the designing of such innovative approaches to achieve theranostic therapy [137]. In one the interesting study glutathione-responsive NPs were developed by assuming that the site specific and controlled release of pDNA would occur in response to the intracellular glutathione [141]. To make the NPs glutathione responsive, thiolated gelatin was synthesized and two different types of gelatin NPs; cross-linked and non-cross-linked were developed and compared with normal gelatin NPs. The developed NPs revealed stimuli responsive release of encapsulated

FITC-dextran in response to the glutathione in release media. The plasmid DNA expressing green fluorescent protein (EGFP-N1) was loaded into the NPs and the delivery potential of the developed NPs was further accessed in murine fibroblast cells. Fluorescence imaging of cells revealed transfection and protein expression after 6 h which was continued as long as 96 h. This concept was further extended and PEG modified glutathione responsive thiolated gelatin NPs were developed by the same group of authors and this time plasmid DNA encoding the soluble form of the extracellular domain of vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) was loaded in the system [142]. In line with the author's concept PEG modified thiolated gelatin NPs exhibited superior in vitro transfection in human breast adenocarcinoma cells in comparison with plain counterparts. The developed NPs were further tested in nu/nu mice bearing orthotopic human breast adenocarcinoma (MDA-MB-435) xenografts and presented significant suppression of tumor growth. Pangburn et al. reported peptide-functionalized polymersomes composed of poly(1,2-butadiene)-b-poly(ethylene oxide) for the encapsulation and delivery of siRNA. In this work, they utilized PR_b, a fibronectin mimetic peptide targeting specifically the α(5)β(1) integrin. Orai3 gene was targeted for siRNA knockdown. PR_b functionalized polymersomes were capable of inducing cell death in T47D breast cancer cells to a certain extent, while preserving viability of noncancerous MCF10A breast cells. Co-localization studies revealed accumulation of functionalized polymersomes within cellular endosomes and lysosomes as assessed by organelle staining. These functionalized vesicles were found to possess higher transfection efficiency and better safety profile compared to the current commercial siRNA transfection agent [143].

In a different strategy NPs-in-microsphere oral system (NiMOS) consists of type B gelatin NPs encapsulated in PCL microspheres was reported for oral gene delivery [144]. PCL was used strategically to protect the particles from degradation in stomach and deliver the particles to intestine where the PCL is degraded by lipases. In bio-distribution studies, designed NPs revealed high accumulation in stomach after one hour which was shifted predominately to the large intestine after 2 h. The efficacy of fabricated system was further tested with two different reporter plasmids expressing β-galactosidase (CMV-βgal) and EGFP-N1 in Wistar rats. Transgene expression was observed in the small and large intestines in both the plasmids although EGFP-N1 expression was more pronounced in case of NiMOS formulation in comparison with controls.

Medarova et al. developed dual purpose probe for the simultaneous noninvasive imaging and delivery of siRNAs to tumors consisted of magnetic NPs (for MRI) labelled with Cy5.5 dye (for NIRF) and conjugated to a synthetic siRNA duplex. Additionally, the probe was modified with myristoylated polyarginine peptides (MPAP) for targetable intracellular gene delivery. The results of in vivo tracking of these multifunctional MNPs demonstrated simultaneous delivery of siRNA to tumors and imaging of its accumulation by high-resolution magnetic resonance imaging (MRI) and near-infrared in vivo optical imaging (NIRF) [103].

In a recent study by Hu et al. surfactant-free lipo-polymersomes (LPPs) were rationally designed for gene therapy. LPPs consist of cationic lipids and plasmid DNA inside the core encircled by polymeric layer loaded with iron oxide nanoparticles and a neutral lipid shell, thus avoiding undesirable interactions with blood components during circulation. Under the influence of external magnetic field and folate receptor mediated endocytosis, 30–40 times higher accumulation was observed in the cell uptake studies. This synergistic tumor targeting approach resulted in tenfold greater transfection efficiency in the animal models with facilitation of endosomal escape of genetic material.

Cyclodextrins (CDs) have unique ability to form inclusion complexes with guests fitting in their hydrophobic cavity and further chemical functionalization endow them excellent delivery vehicle. The excellency of CD, in the field of gene delivery is reflected by the number of successful reports in which CD was taken as key constituent in the designing of delivery vehicle. Zhang et al. reported novel core-shell structured nanoassemblies, as a new generation of multifunctional nanocarriers, for simultaneous drug delivery and gene therapy. The nanoassemblies were formed by synthesizing cyclodextrin-polyethyleneimine and Poly (β-benzyl L-aspartate) (PBLA) as a host and guest polymers, respectively in which the hydrophobic core served as a nano-container to load and release the hydrophobic drugs (Dexamethasone), while plasmid DNA was condensed at the positively charged hydrophilic shell. In line with the author's hypothesis, the developed nano-assemblies exhibited excellent transfection and lower cytotoxicity in MC3T3-E1 osteoblast cells. Furthermore, presence of functionally active amine groups at the periphery supposed to provide additional functionalities to proposed system [145]. Cyclodextrin based systems, ornamented with different varieties of ligands, have also been reported for targeted gene delivery. Cyclodextrin based nanoparticle formulation decorated with transferrin (Tf) was reported for targeted delivery of genetic material to cancer cells [146, 147]. Khalil et al. developed multifunctional envelope-type nano-device (MEND) that was supposed to mimic an envelope-type virus. Luciferase expression plasmid DNA was packaged in the core of these particles and further decorated with octaarginine peptide which mediates the internalization of these particles via macropinocytosis simultaneously avoiding the lysosomal degradation. The MEND developed during the study exhibited equivalent transfection and lower cytotoxicity in comparison with adenovirus. Furthermore, topical application of MEND particles containing constitutively active bone morphogenetic protein (BMP) type IA receptor (caBmpr1a) gene exhibited significant impact on hair growth in ICR mice in vivo [148]. The concept of developing MEND [149–151], using octaarginine and/or other fusogenic peptide was also successfully explored for gene delivery in different reports [152–156]. A summary of different delivery strategy using multifunctional nano-carriers has been presented in Table 15.3.

Table 15.3 Different approaches of providing multifunctionality to polymeric nano-carriers for gene delivery

Entrapped moiety	Nanotechnology platform	Multifunctionality	References
Paclitaxel and DNA	(PCL-g-PDMAEMA) Poly(3 caprolactone)-graft-poly(2 (dimethylamino) ethyl methacrylate) based NPs	Dual delivery of drug and DNA for synergistic effect against tumors	[157]
Plasmid DNA	Poly(L-ornithine) NPs	Functionalization with galactose and fusigenic peptide (mHA2) to deliver DNA to hepatocytes through ASGP receptor recognition	[158]
Plasmid DNA	Nanocomplexes based on CyD	Surface modification with PEG and transferrin for cancer targeting	[147]
siRNA	Nanocomplexes based on PEI	Surface engineering with PEG and RGD peptide to target VEGF R2 receptors overexpressed on cancer cells	[159]
Plasmid DNA	Nanocomplexes based on PEI	RGD/HIV-1 Tat peptides functionalization for endolysosomal escape	[160]
Plasmid DNA	Gelatine based NPs	Surface functionalization with epidermal growth factor receptor (EGFR) -targeting peptide overexpressed in human pancreatic adenocarcinoma cells (Panc-1)	[161]
Plasmid DNA	NPs composed of PLGA-PEGMA block copolymers	Folate functionalization to target folate receptors overexpressed over tumor cells	[162]
Paclitaxel and siRNA	PLGA NPs	Dual delivery of drug and siRNA along with surface functionalization with biotin for effective silencing of the MDR1 gene that encodes for P-gp	[163]
DGKα	Nanocomplexes based on PEI	Surface functionalization with CD3 single chain antibody (scAbCD3) along with SPIO for MRI imaging	[164]

15.6 Multifunctional Polymeric Nanoparticles for Other Disorders

Apart from the cancer and gene delivery multifunctional nano-carriers have also been explored as a platform technology to a variety of diseases like brain disorders and liver diseases to improve the therapeutic performance.

15.6.1 Multifunctional Polymeric Nanoparticles for Targeted Brain Delivery

Brain targeting is one of the most challenging and arduous area of research as the presence of blood–brain barrier, comprising cerebral endothelial cells with tight junctions, hinders the availability of bio-actives to the brain. In contemplation of treating CNS related disorders drug has to enter CNS by crossing the resilient blood–brain barrier. Generally drugs or any other molecules irrespective of their size cannot enter brain due to the effective prevention of entry of any kind of molecules into brain. Many strategies have been identified and applied in delivering therapeutics across the BBB of which direct drug conjugation and encapsulation of therapeutic moieties in nano-carriers are prominently studied. Direct drug conjugation includes modification of drug molecules so as to mimic the endogenous ligand while preserving its therapeutic activity or combining with other molecules via bond formation so as to achieve either carrier-mediated/receptor-mediated transport of respective therapeutic agents [165]. A variety of nano-carriers like polymeric NPs, liposomes, solid lipid nanoparticles, albumin NPs, dendrimers, nano-emulsions, and nano-suspensions have been explored for the effective delivery [166]. Improved drug transport through BBB by using nano-carriers have been hypothesized by a variety of mechanisms which includes [167–170]: (1) Adsorption and retention in the brain capillaries which maintains the concentration gradient and further facilitate the drug transport across the BBB, (2) Solubilization and fluidization of membrane lipids of brain endothelial cells by the surfactant nature of certain components of nano-carriers, (3) Opening of tight junctions between brain endothelial cells, (4) Transcytosis or endocytosis of nano-carriers.

Nanoparticulate system comprising Poly(n-butylcyanoacrylate) coated with polysorbate 80 was successfully tested for many types of drug molecules like hexapeptide dalargin, the dipeptide kytorphin, loperamide, tubocurarine, and DOX for treatment of different diseases/disorders like Alzheimer's disease, multiple sclerosis, Parkinson's disease, inflammation, and brain cancer as well [168, 169, 171–178]. Kreuter et al. investigated the role of apolipoproteins (AII, B, CII, E, or J) in transport of drug molecules across the blood–brain barrier by using the same concept taking dalargin and loperamide as model drugs. Antinociceptive threshold measurements in mice by tail flick test following IV administration of different formulations demonstrated significantly higher efficacy in vivo following the coating of polysorbate 80

and/or apolipoprotein E. After getting attached with the apolipoprotein B and/or E, NPs resemble with the lipoprotein particles of biological system and actively taken up by the lipoprotein receptors present on the capillary endothelial cells of blood–brain barrier. The similar effect could be observed with different polysorbates too [167, 174, 179]. Similar formulation strategy was reported by Wilson et al. for the delivery of reversible cholinesterase inhibitor, rivastigmine and confirmed the improved delivery potential of polysorbate 80 coated NPs [180]. Increased levels of different metals like iron, zinc, copper, mercury, and aluminium in different parts of brain is also observed in Alzheimer's disease [181, 182]. These accumulated metals may produce significant oxidative stress by producing free radicals ultimately leading to neurodegeneration. Neurons are especially prone to attacks by free radicals because of the presence of low quantities of natural antioxidant glutathione and high quantities of polyunsaturated fatty acids [183]. Metal chelators are special type of molecules that can bind to excess quantities of metals and thus facilitating their removal by which oxidative stress of brain can be relieved. Many free radical scavengers like vitamin E (α-tocopherol), selegiline, and *Ginkgo biloba* extract EGb 761(Tanakan) have been reported to be used as a targeting strategy with promising efficacy [182].

Brain tumor is another major research area of concern in which crossing the BBB is the major hurdle. After crossing the BBB, targeting of the delivery vehicle specifically to the tumor without causing systemic toxicity is next level challenge. The purpose of enhancing permeation with improved efficacy and reduced cytotoxicity can be effectively achieved by encapsulating the bio-actives in functionalized nano-carriers. Functionalized nano-carriers can cross blood–brain barrier quite easily by aforementioned mechanisms and can get internalized especially in cancerous cells where they can exert an action. Besides the conventional therapy, photodynamic therapy has also been explored for the successful treatment of brain tumors. Photodynamic therapy actually involves delivering of a photosensitizing molecule into tumor cells followed by activation by specific wavelength of visible or near-infrared light. This activation makes the photosensitizing molecule to produce singlet reactive oxygen species (1O2) which is cytotoxic and can kill the tumor cells permanently by creating inflammation and hypoxic conditions [184, 185]. The efficiency of photosensitizing molecule to produce reactive oxygen species can be determined by anthracene-9, l0-dipropionic acid, disodium salt (ADPA) method. If the decrease in fluorescence intensity is observed upon mixing of sample with ADPA followed by irradiation at 650 nm, it indicates the generation of free oxygen species which reacts with ADPA in an irreversible fashion. Tang et al. reported polyacrylamide based functionalized NPs for the photodynamic therapy to treat brain tumors by taking methylene blue as a photosensitizer. The developed NPs demonstrated high release of reactive oxygen species and thus higher cytotoxicity to cancerous cells [185].

Reddy et al. developed multifunctional polymeric NPs for the purpose of brain targeting along with imaging. The system is basically polyacrylamide NPs encapsulated with photofrin for the therapeutic benefit while the iron oxide was taken as a magnetic resonance contrasting agent. Furthermore, the NPs were functionalized

Table 15.4 Different strategies of multifunctionality for brain targeting

Active ingredient	Nanotechnology platform	Multifunctionality	References
Dalargin, Loperamide, Rivastigmine, Kyotorphin, Tubocurarine, DOX, Temozolomide, Zidovudine & Lamivudine, clioquinol	Poly(n-butylcyano-acrylate) NPs	Polysorbate 80 coating which make the apolipoprotein B and/or E to bind with NPs resembling native lipoprotein molecules	[167, 169, 171, 177, 180, 188–190]
^{14}C radiolabelling	Polycyanoacrylate NPs	PEGylation for enhanced plasma circulation and brain diffusion	[191]
Methylene blue	Polyacrylamide NPs	Methylene blue is a photosensitizer simultaneously serving the purpose of imaging	[185]
Vasoactive intestinal peptide	Poly (ethylene glycol)-poly (lactic acid) NPs	Surface functionalization with wheat germ agglutinin (WGA)	[192]
Loperamide	Human serum albumin NPs	Surface functionalization with transferrin/transferring antibodies to promote the entry through BBB	[193]
Photofrin	Polyacrylamide NPs	Surface functionalization with F3 and co-encapsulation of iron oxide as MRI contrast agent	[119]

with F3 peptide, which is a 31 amino acid peptide with cell penetrating properties and high specificity for tumor cells [186, 187]. Improved efficacy was observed in MDA-435 cell lines while the greater accumulation of surface functionalized NPs was confirmed by magnetic resonance imaging. Based on the positive findings, these multifunctional NPs were found beneficial in terms of providing more factual results [119]. A summary of different strategies of multifunctionality for brain targeting is shown in Table 15.4.

15.6.2 Multifunctional Polymeric Nano-Carriers for Liver Targeting

Liver is the major organ involved in metabolism of drugs and liver cancer may triggers many severe complications. Hepatitis B virus (HBV) is the major cause of liver cancer followed by Hepatitis C virus (HCV) and some environmental factors that includes aflatoxin B1, smoking, drinking and mycotoxins [194–196]. HBV acts directly and involves damage of hepatocytes and DNA inside these cells but unlike

HBV, HCV acts indirectly by initiating cirrhosis. Current liver cancer therapy predominantly relies on chemotherapy; however, short circulation half-life and systemic toxicity due to the non-selectivity are the major limitations. A variety of molecules like galactose, glycyrrhizin, sugars, folic acid, and antibodies have been found effective as targeting ligand for liver cancer. Asialoglycoprotein (ASGP) receptor, which is also known as galactose receptor, has been reported to be overexpressed specifically in hepatocytes of liver [197] which are capable of recognizing terminal β-D-galactose and *N*-acetylgalactosamine residues and hence can be utilized as potential targeting ligand to ensure precise drug delivery to liver [198]. These receptors allow the specific binding of galactose functionalized systems and readily get internalized via receptor mediated endocytosis [199]. Liang et al. developed galactosamine functionalized poly(g-glutamic acid)-poly(lactide) NPs for targeted delivery of PTX to liver and found that modification with galactosamine demonstrated significantly higher cell growth inhibition potential in HepG2 cell lines. Moreover, bio-distribution studies in normal and hepatoma-tumor-bearing nude mice after rhodamine labelling, demonstrated higher accumulation in liver and tumor regions. Furthermore, galactosamine functionalized NPs were found much effective in tumor growth suppression and confirmed the specific liver targeting potential of galactosamine functionalized nano-carriers via asialoglycoprotein receptor mediated endocytosis [200]. The concept of liver targeting by using galactose/galactosamine has been supported in a variety of reports for the delivery of all trans-retinoic acid by poly(L-lactic acid) NPs [201], gene delivery to hepatocytes by poly(ethylene glycol)-chitosan-graft-polyethylenimine [202], fluorescent labeled micelles as a liver targeting drug carrier [203]. Although this strategy was found quite effective in liver targeting, reduced density and activity of ASGP receptors in patients with liver disease due to reduced binding capability of ASGP receptors may limit its use. To overcome this limitation other targeting moieties, glycyrrhizin and/or glycyrrhizinic acid, came into picture [204, 205]. Glycyrrhizin (GL) and/or glycyrrhizinic acid (GA) are the main component in roots of liquorice having wide range of therapeutic applications such as anti-allergic, anti-inflammatory, and anti-hepatotoxic effects and gelling agent in cosmetics [206]. It was recognized that rat hepatocytes contains the more specific binding receptors for GL and GA [207–209] and promote the internalization of GL/GA functionalized nano-carriers through receptor mediated endocytosis [210]. Lin et al. evaluated the hepatocyte targeting potential of glycyrrhizin functionalized chitosan NPs by using adriamycin as a model drug. Glycyrrhizin modified and rhodamine B isothiocyanate (RBITC) loaded chitosan NPs demonstrated 4.9 times higher accumulation of GL-CH-NPs in hepatocytes than endothelial cells while unmodified CS-NPs exhibited almost equal uptake in both hepatocytes and endothelial cells. CLSM studies revealed higher internalization of GL-CS-NPs into cytoplasm of hepatocytes and supported the strategy for effective liver or more specifically hepatocyte targeting [210]. Tian et al. developed Glycyrrhetinic acid-modified chitosan/poly(ethylene glycol) NPs (GA-CS/PEG) and used single-photon emission computed tomography (SPECT) technique for noninvasive imaging and quantitative distribution of functionalized NPs. SPECT analysis demonstrated much higher localization of GA-CS/PEG in

liver immediately after administration of 99mTc labelled GA-CS/PEG NPs in comparison with other organs like kidney and bladder and other unmodified formulation. Cell uptake studies in human hepatocytes (QGY-7703 cells) demonstrated much higher fluorescence in case of GA modification in comparison with unmodified NPs. Furthermore, promising cytotoxicity and antitumor efficacy of DOX loaded NPs established the potential of proposed strategy to achieve liver targeting [205]. Among the different targeting moieties folic acid has also been recognized as potential target as the folate receptors have been reported to be overexpressed over the cancerous cells [34, 211]. This targeting strategy was used by Zhang et al. to develop multifunctional polymeric micelles based on polyethylene glycol-block-poly(ε-caprolactone) (PEG-PCL) by taking sorafenib as a potential anticancer agent. The micelles were surface functionalized with folic acid to have the advantage of folate receptor mediated uptake and simultaneously loaded with SPIO NPs as an MRI contrast agent. Prussian blue staining stipulated the uptake of folate targeted micelles into human hepatic carcinoma (HepG2) cells. In addition it was perceived that uptake of folate functionalized micelles got reduced upon inclusion of free folate to HepG2 cells which confirmed the involvement of folate receptors in cellular uptake of developed micelles. Furthermore, targeted micelles were found to be effective in inhibiting the proliferation and enhancing the apoptosis of HepG2 cells [212]. Table 15.5 summarizes the different strategies of multifunctionality which have been explored for the purpose of liver targeting.

15.7 Conclusion and Future Outlook

The solitary purpose of NPs is tremendously changing from simply ferrying cargo to smart carriers with all in one approach which is supposed to target any disease or any organ. These nanoparticulate platforms are the need of the hour to ensure the effective therapy along with live imaging without aggravating the unwanted side effects to the normal tissues. Despite the endless benefits offered by multifunctional polymeric nano-carriers, there are several challenges which need to be addressed to bring them from bench to bedside. Crowded nature of multifunctional polymeric nano-carriers may interfere with the selective recognition and uptake of these carriers, which may ultimately lead to toxicity. Thus, there is a strong need to study the effect of multifunctionality on selectivity and uptake in more detail to eliminate all the possible side effect as much as possible. This can be improved by using surface engineering to develop polymeric nano-carriers which can simultaneously bind to the multiple target sites and preferentially localize the therapeutics and contrast agents to target site. This improved targeting approach can further reduce the unnecessary drug load by preferential accumulation of therapeutics to target site. Another area which needs to be worked upon is the development of biologically active polymers as building blocks in nano-carriers fabrication. In this area, polymer drug or polymers with inherent fluorescence can be developed to directly fabricate the NPs which will not only reduce the burden of multifunctionality but also open a new era of research.

Table 15.5 Different approaches of multifunctionality for liver targeting

Active ingredient	Nanotechnology platform	Multifunctionality	References
All trans-retinoic acid	Poly (L-lactic acid) NPs	Surface functionalization with galactose to target ASGP receptors on hepatocytes	[201]
DNA	Chitosan NPs	Surface galactocylation to target ASGP receptors	[213]
DNA	Chitosan NPs Folate-chitosan-DNA NPs	Surface functionalization by folic acid to target folate receptors overexpressed on cancerous cells	[214]
Paclitaxel	Poly(g-glutamic acid)-poly(lactide) NPs	Surface functionalization with galactosamine to target ASGP receptors	[200]
Adriamycin	Chitosan NPs	Surface functionalization with glycyrrhizin	[210]
Gadolinium	PLA-PEG-PLL NPs	Surface functionalization with Anti-VEGF along with gadolinium as MRI contrast agent	[215]
DOX	PEG-PCL micelles	Surface modification with folic acid along with co-encapsulation of SPIO for simultaneous MRI imaging	[216]
DOX	PEG-PLA micelles	Surface functionalization with cRGD to target αvβ3 integrins on tumor endothelial cells with simultaneous encapsulation of SPIO for MRI scanning	[104]
Sorafenib	PEG-PCL micelles	Surface functionalization with folic acid to target folate receptors along with SPION for MRI imaging	[212]

References

1. Farokhzad OC, Langer R (2009) Impact of nanotechnology on drug delivery. ACS Nano 3:16–20. doi:10.1021/nn900002m
2. Singh R, Lillard JW Jr (2009) Nanoparticle-based targeted drug delivery. Exp Mol Pathol 86:215–223. doi:10.1016/j.yexmp.2008.12.004
3. Agrawal AK, Harde H, Thanki K, Jain S (2013) Improved stability and antidiabetic potential of insulin containing folic acid functionalized polymer stabilized multilayered liposomes following oral administration. Biomacromolecules 15:350–360. doi:10.1021/bm401580k
4. Jain S, Indulkar A, Harde H, Agrawal AK (2014) Oral mucosal immunization using glucomannosylated bilosomes. J Biomed Nanotechnol 10:932–947. doi:10.1166/jbn.2014.1800
5. Jain S, Harde H, Indulkar A, Agrawal AK (2013) Improved stability and immunological potential of tetanus toxoid containing surface engineered bilosomes following oral administration. Nanomed Nanotechnol Biol Med 10:431–440. doi:10.1016/j.nano.2013.08.012
6. Jain S, Sharma JM, Agrawal AK, Mahajan RR (2013) Surface stabilized efavirenz nanoparticles for oral bioavailability enhancement. J Biomed Nanotechnol 9:1862–1874. doi:10.1166/jbn.2013.1683

7. Harde H, Agrawal AK, Jain S (2014) Development of stabilized glucomannosylated chitosan nanoparticles using tandem crosslinking method for oral vaccine delivery. Nanomedicine (Lond), 1–19. doi:10.2217/nnm.13.225
8. Agrawal A, Gupta P, Khanna A, Sharma R, Chandrabanshi H, Gupta N, Patil U, Yadav S (2010) Development and characterization of in situ gel system for nasal insulin delivery. Pharmazie 65:188–193. doi:10.1691/ph.2010.9188
9. Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE (2001) Biodegradable polymeric nanoparticles as drug delivery devices. J Control Release 70:1–20. doi:10.1016/S0168-3659(00)00339-4
10. Sanvicens N, Marco MP (2008) Multifunctional nanoparticles–properties and prospects for their use in human medicine. Trends Biotechnol 26:425–433. doi:10.1016/j.tibtech.2008.04.005
11. Pillai O, Panchagnula R (2001) Polymers in drug delivery. Curr Opin Chem Biol 5:447–451. doi:10.1016/S1367-5931(00)00227-1
12. Lee KY, Mooney DJ (2012) Alginate: properties and biomedical applications. Prog Polym Sci 37:106–126. doi:10.1016/j.progpolymsci.2011.06.003
13. Sun G, Shen YI, Ho CC, Kusuma S, Gerecht S (2010) Functional groups affect physical and biological properties of dextran-based hydrogels. J Biomed Mater Res A 93:1080–1090. doi:10.1002/jbm.a.32604
14. Serefoglou E, Oberdisse J, Staikos G (2007) Characterization of the soluble nanoparticles formed through coulombic interaction of bovine serum albumin with anionic graft copolymers at low pH. Biomacromolecules 8:1195–1199. doi:10.1021/bm061094t
15. Chopra S, Mahdi S, Kaur J, Iqbal Z, Talegaonkar S, Ahmad FJ (2006) Advances and potential applications of chitosan derivatives as mucoadhesive biomaterials in modern drug delivery. J Pharm Pharmacol 58:1021–1032. doi:10.1211/jpp.58.8.0002
16. Yin L, Ding J, He C, Cui L, Tang C, Yin C (2009) Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery. Biomaterials 30:5691–5700. doi:10.1016/j.biomaterials.2009.06.055
17. Agrawal AK, Das M, Jain S (2012) In situ gel systems as 'smart' carriers for sustained ocular drug delivery. Expert Opin Drug Deliv 9:383–402. doi:10.1517/17425247.2012.665367
18. Choudhary H, Agrawal A, Malviya R, Yadav S, Jaliwala Y, Patil U (2010) Evaluation and optimization of preparative variables for controlled-release floating microspheres of levodopa/carbidopa. Pharmazie 65:194–198. doi:10.1691/ph.2010.9288
19. Kopeček J, Ulbrich K (1983) Biodegradation of biomedical polymers. Prog Polym Sci 9:1–58
20. Panyam J, Labhasetwar V (2003) Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev 55:329–347. doi:10.1016/S0169-409X(02)00228-4
21. Wu K-J, Wu C-S, Chang J-S (2007) Biodegradability and mechanical properties of polycaprolactone composites encapsulating phosphate-solubilizing bacterium Bacillus sp. PG01. Process Biochem 42:669–675
22. Richard A, Margaritis A (2001) Poly (glutamic acid) for biomedical applications. Crit Rev Biotechnol 21:219–232. doi:10.1080/07388550108984171
23. Jain S, Patil SR, Swarnakar NK, Agrawal AK (2012) Oral delivery of doxorubicin using novel polyelectrolyte-stabilized liposomes (layersomes). Mol Pharm 9:2626–2635. doi:10.1021/mp300202c
24. Brannon-Peppas L, Blanchette JO (2012) Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev 64:206–212. doi:10.1016/j.addr.2012.09.033
25. Brigger I, Dubernet C, Couvreur P (2002) Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev 54:631–651. doi:10.1016/S0169-409X(02)00044-3
26. Das M, Mishra D, Maiti T, Basak A, Pramanik P (2008) Bio-functionalization of magnetite nanoparticles using an aminophosphonic acid coupling agent: new, ultradispersed, iron-oxide folate nanoconjugates for cancer-specific targeting. Nanotechnology 19:415101. doi:10.1088/0957-4484/19/41/415101

27. Cascante M, Centelles JJ, Veech RL, Lee W-NP, Boros LG (2000) Role of thiamin (vitamin B-1) and transketolase in tumor cell proliferation. Nutr Cancer 36:150–154. doi:10.1207/S15327914NC3602_2
28. Park JW, Benz CC, Martin FJ (2004) Future directions of liposome-and immunoliposome-based cancer therapeutics. Semin Oncol 31(6 Suppl 13):196–205
29. Kumari A, Yadav SK, Yadav SC (2010) Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf B: Biointerfaces 75:1–18. doi:10.1016/j.colsurfb.2009.09.001
30. Jain AK, Das M, Swarnakar NK, Jain S (2011) Engineered PLGA nanoparticles: an emerging delivery tool in cancer therapeutics. Crit Rev Ther Durg 28:1–45. doi:10.1615/CritRevTherDrugCarrierSyst.v28.i1.10
31. Shenoy D, Little S, Langer R, Amiji M (2005) Poly (ethylene oxide)-modified poly (β-amino ester) nanoparticles as a pH-sensitive system for tumor-targeted delivery of hydrophobic drugs: part 2. In vivo distribution and tumor localization studies. Pharm Res 22:2107–2114
32. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM (1999) Polymeric systems for controlled drug release. Chem Rev 99:3181–3198. doi:10.1021/cr940351u
33. Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, Richie JP, Langer R (2006) Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo. Proc Natl Acad Sci U S A 103:6315–6320. doi:10.1073/pnas.0601755103
34. Antony A (1992) The biological chemistry of folate receptors. Blood 79:2807–2820
35. Vidal JM, Koulibaly M, Jost JL, Duron JJ, Chigot JP, Vayre P, Aurengo A, Legrand JC, Rosselin G, Gespach C (1998) Differential transferrin receptor density in human colorectal cancer: a potential probe for diagnosis and therapy. Int J Oncol 13:871–875. doi:10.3892/ijo.13.4.871
36. Rusch V, Klimstra D, Venkatraman E, Pisters PW, Langenfeld J, Dmitrovsky E (1997) Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression. Clin Cancer Res 3:515–522
37. Korc M, Chandrasekar B, Yamanaka Y, Friess H, Buchier M, Beger HG (1992) Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor alpha. J Clin Invest 90:1352–1360. doi:10.1172/jci116001
38. Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F (2005) Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. Breast Cancer Res 7:R788–R795. doi:10.1186/bcr1304
39. Lewis GD, Lofgren JA, McMurtrey AE, Nuijens A, Fendly BM, Bauer KD, Sliwkowski MX (1996) Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. Cancer Res 56:1457–1465
40. Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. Nat Rev Cancer 7:79–94. doi:10.1038/nrc2069
41. Rozengurt E, Guha S, Sinnett-Smith J (2002) Gastrointestinal peptide signalling in health and disease. Eur J Surg 587:23–38
42. Szepeshazi K, Schally AV, Nagy A, Halmos G (2005) Inhibition of growth of experimental human and hamster pancreatic cancers in vivo by a targeted cytotoxic bombesin analog. Pancreas 31:275–282
43. Liu G, Duranteau L, Carel J-C, Monroe J, Doyle DA, Shenker A (1999) Leydig-cell tumors caused by an activating mutation of the gene encoding the luteinizing hormone receptor. N Engl J Med 341:1731–1736. doi:10.1056/NEJM199912023412304
44. Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. Nature 365:649–651. doi:10.1038/365649a0
45. Kukowska-Latallo JF, Candido KA, Cao Z, Nigavekar SS, Majoros IJ, Thomas TP, Balogh LP, Khan MK, Baker JR (2005) Nanoparticle targeting of anticancer drug improves therapeutic

response in animal model of human epithelial cancer. Cancer Res 65:5317–5324. doi:10.1158/0008-5472.CAN-04-3921
46. Iinuma H, Maruyama K, Okinaga K, Sasaki K, Sekine T, Ishida O, Ogiwara N, Johkura K, Yonemura Y (2002) Intracellular targeting therapy of cisplatin-encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer. Int J Cancer 99:130–137. doi:10.1002/ijc.10242
47. Ishida O, Maruyama K, Tanahashi H, Iwatsuru M, Sasaki K, Eriguchi M, Yanagie H (2001) Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo. Pharm Res 18:1042–1048. doi:10.1023/A:1010960900254
48. Ekblom P, Thesleff I, Lehto VP, Virtanen I (1983) Distribution of the transferrin receptor in normal human fibroblasts and fibrosarcoma cells. Int J Cancer 31:111–117. doi:10.1002/ijc.2910310118
49. Li J, Ji J, Holmes LM, Burgin KE, Barton LB, Yu X, Wagner TE, Wei Y (2004) Fusion protein from RGD peptide and Fc fragment of mouse immunoglobulin G inhibits angiogenesis in tumor. Cancer Gene Ther 11:363–370. doi:10.1038/sj.cgt.7700707
50. Ruoslahti E (1994) Cell adhesion and tumor metastasis. Princess Takamatsu Symp 24:99–105
51. Peer D, Margalit R (2004) Tumor-targeted hyaluronan nanoliposomes increase the antitumor activity of liposomal doxorubicin in syngeneic and human xenograft mouse tumor models. Neoplasia 6(4):343–353, doi:10.1593/neo.03460
52. Hu Z, Sun Y, Garen A (1999) Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. Proc Natl Acad Sci U S A 96:8161–8166. doi:10.1073/pnas.96.14.8161
53. Upadhyay KK, Le Meins JF, Misra A, Voisin P, Bouchaud V, Ibarboure E, Schatz C, Lecommandoux S (2009) Biomimetic doxorubicin loaded polymersomes from hyaluronan-block-poly(gamma-benzyl glutamate) copolymers. Biomacromolecules 10:2802–2808. doi:10.1021/bm9006419
54. Upadhyay KK, Bhatt AN, Mishra AK, Dwarakanath BS, Jain S, Schatz C, Le Meins JF, Farooque A, Chandraiah G, Jain AK, Misra A, Lecommandoux S (2010) The intracellular drug delivery and anti tumor activity of doxorubicin loaded poly(gamma-benzyl L-glutamate)-b-hyaluronan polymersomes. Biomaterials 31:2882–2892. doi:10.1016/j.biomaterials.2009.12.043
55. Upadhyay KK, Mishra AK, Chuttani K, Kaul A, Schatz C, Le Meins JF, Misra A, Lecommandoux S (2012) The in vivo behavior and antitumor activity of doxorubicin-loaded poly(gamma-benzyl l-glutamate)-block-hyaluronan polymersomes in Ehrlich ascites tumor-bearing BalB/c mice. Nanomedicine 8:71–80. doi:10.1016/j.nano.2011.05.008
56. Upadhyay KK, Bhatt AN, Castro E, Mishra AK, Chuttani K, Dwarakanath BS, Schatz C, Le Meins JF, Misra A, Lecommandoux S (2010) In vitro and in vivo evaluation of docetaxel loaded biodegradable polymersomes. Macromol Biosci 10:503–512. doi:10.1002/mabi.200900415
57. Pang Z, Gao H, Yu Y, Guo L, Chen J, Pan S, Ren J, Wen Z, Jiang X (2011) Enhanced intracellular delivery and chemotherapy for glioma rats by transferrin-conjugated biodegradable polymersomes loaded with doxorubicin. Bioconjug Chem 22:1171–1180. doi:10.1021/bc200062q
58. Gabizon AA (2001) Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. Cancer Invest 19:424–436
59. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2:751–760. doi:10.1038/nnano.2007.387
60. Goldenberg MM (1999) Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. Clin Ther 21:309–318. doi:10.1016/S0149-2918(00)88288-0
61. Martiny-Baron G, Marmé D (1995) VEGF-mediated tumour angiogenesis: a new target for cancer therapy. Curr Opin Biotechnol 6:675–680. doi:10.1016/0958-1669(95)80111-1

62. Allen TM (2002) Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2:750–763. doi:10.1038/nrc903
63. Carter P (2001) Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 1:118–129. doi:10.1038/35101072
64. Arnold DM, Dentali F, Crowther MA, Meyer RM, Cook RJ, Sigouin C, Fraser GA, Lim W, Kelton JG (2007) Systematic review: efficacy and safety of rituximab for adults with idiopathic thrombocytopenic purpura. Ann Intern Med 146:25–33. doi:10.7326/0003-4819-146-1-200701020-00006
65. Trail P, Willner D, Lasch S, Henderson A, Hofstead S, Casazza A, Firestone R, Hellstrom I, Hellstrom K (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science 261:212–215. doi:10.1126/science.8327892
66. Tolcher AW, Sugarman S, Gelmon KA, Cohen R, Saleh M, Isaacs C, Young L, Healey D, Onetto N, Slichenmyer W (1999) Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. J Clin Oncol 17:478–478
67. White RR, Sullenger BA, Rusconi CP (2000) Developing aptamers into therapeutics. J Clin Invest 106:929–934. doi:10.1172/JCI11325
68. Debets MF, Leenders WP, Verrijp K, Zonjee M, Meeuwissen SA, Otte-Höller I, van Hest J (2013) Nanobody-functionalized polymersomes for tumor-vessel targeting. Macromol Biosci 13:938–945, doi:10.1002/mabi.201300039
69. Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62. doi:10.1126/science.1104819
70. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6:273–286. doi:10.1038/nrd2115
71. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA (2003) A randomized trial of bevacizumab, an anti–vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 349:427–434. doi:10.1056/NEJMoa021491
72. Cobleigh MA, Langmuir VK, Sledge GW, Miller KD, Haney L, Novotny WF, Reimann JD, Vassel A (2003) A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. Semin Oncol 30(5 Suppl 16):117–124
73. Mayer RJ (2004) Two steps forward in the treatment of colorectal cancer. N Engl J Med 350:2406–2408. doi:10.1056/NEJMe048098
74. Emanuel S, Gruninger RH, Fuentes-Pesquera A, Connolly PJ, Seamon JA, Hazel S, Tominovich R, Hollister B, Napier C, D'Andrea MR (2004) A vascular endothelial growth factor receptor-2 kinase inhibitor potentiates the activity of the conventional chemotherapeutic agents paclitaxel and doxorubicin in tumor xenograft models. Mol Pharmacol 66:635–647. doi:10.1124/mol.104.000638
75. Ma L, Francia G, Viloria-Petit A, Hicklin DJ, du Manoir J, Rak J, Kerbel RS (2005) In vitro procoagulant activity induced in endothelial cells by chemotherapy and antiangiogenic drug combinations: modulation by lower-dose chemotherapy. Cancer Res 65:5365–5373. doi:10.1158/0008-5472.CAN-04-3156
76. Tuettenberg J, Grobholz R, Korn T, Wenz F, Erber R, Vajkoczy P (2005) Continuous low-dose chemotherapy plus inhibition of cyclooxygenase-2 as an antiangiogenic therapy of glioblastoma multiforme. J Cancer Res Clin Oncol 131:31–40. doi:10.1007/s00432-004-0620-5
77. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342. doi:10.1056/NEJMoa032691
78. Kabbinavar FF, Hambleton J, Mass RD, Hurwitz HI, Bergsland E, Sarkar S (2005) Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. J Clin Oncol 23:3706–3712. doi:10.1200/JCO.2005.00.232
79. McCarthy M (2003) Antiangiogenesis drug promising for metastatic colorectal cancer. Lancet 361:1959. doi:10.1016/S0140-6736(03)13603-3

80. Sengupta S, Eavarone D, Capila I, Zhao G, Watson N, Kiziltepe T, Sasisekharan R (2005) Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system. Nature 436:568–572. doi:10.1038/nature03794
81. Donnenberg VS, Donnenberg AD (2005) Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. J Clin Pharmacol 45:872–877. doi:10.1177/0091270005276905
82. Bradley G, Juranka PF, Ling V (1988) Mechanism of multidrug resistance. BBA-Rev Cancer 948:87–128. doi:10.1016/0304-419X(88)90006-6
83. Harris AL, Hochhauser D (1992) Mechanisms of multidrug resistance in cancer treatment. Acta Oncol 31:205–213. doi:10.3109/02841869209088904
84. Hayes JD, Pulford DJ (1995) The glut athione S-transferase supergene family: regulation of GST and the contribution of the lsoenzymes to cancer chemoprotection and drug resistance part I. Crit Rev Biochem Mol Biol 30:445–520. doi:10.3109/10409239509083491
85. Adams J, Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26:1324–1337. doi:10.1038/sj.onc.1210220
86. Ferreira C, Tolis C, Giaccone G (1999) p53 and chemosensitivity. Ann Oncol 10:1011–1021. doi:10.1023/A:1008361818480
87. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP–dependent transporters. Nat Rev Cancer 2:48–58. doi:10.1038/nrc706
88. Takara K, Sakaeda T, Okumura K (2006) An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. Curr Pharm Des 12:273–286. doi:10.2174/138161206775201965
89. Ferry D, Traunecker H, Kerr D (1996) Clinical trials of P-glycoprotein reversal in solid tumours. Eur J Cancer 32:1070–1081. doi:10.1016/0959-8049(96)00091-3
90. Oh KT, Baik HJ, Lee AH, Oh YT, Youn YS, Lee ES (2009) The reversal of drug-resistance in tumors using a drug-carrying nanoparticular system. Int J Mol Sci 10:3776–3792. doi:10.3390/ijms10093776
91. Palmeira A, Sousa E, Vasconcelos MH, Pinto MM (2012) Three decades of P-gp inhibitors: skimming through several generations and scaffolds. Curr Med Chem 19:1946–2025. doi:10.2174/092986712800167392
92. Davis ME, Shin DM (2008) Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat Rev Drug Discov 7:771–782. doi:10.1038/nrd2614
93. Emilienne Soma C, Dubernet C, Bentolila D, Benita S, Couvreur P (2000) Reversion of multidrug resistance by co-encapsulation of doxorubicin and cyclosporin A in polyalkylcyanoacrylate nanoparticles. Biomaterials 21:1–7. doi:10.1016/S0142-9612(99)00125-8
94. Patil Y, Sadhukha T, Ma L, Panyam J (2009) Nanoparticle-mediated simultaneous and targeted delivery of paclitaxel and tariquidar overcomes tumor drug resistance. J Control Release 136:21–29. doi:10.1016/j.jconrel.2009.01.021
95. Hughes CS, Vaden SL, Manaugh CA, Price GS, Hudson L (1998) Modulation of doxorubicin concentration by cyclosporin A in brain and testicular barrier tissues expressing P-glycoprotein in rats. J Neurooncol 37:45–54. doi:10.1023/A:1005900908540
96. Erlichman C, Moore M, Thiessen JJ, Kerr IG, Walker S, Goodman P, Bjarnason G, DeAngelis C, Bunting P (1993) Phase I pharmacokinetic study of cyclosporin A combined with doxorubicin. Cancer Res 53:4837–4842
97. Kim JS, Rieter WJ, Taylor KM, An H, Lin W, Lin W (2007) Self-assembled hybrid nanoparticles for cancer-specific multimodal imaging. J Am Chem Soc 129:8962–8963. doi:10.1021/ja073062z
98. Rieter WJ, Kim JS, Taylor KM, An H, Lin W, Tarrant T, Lin W (2007) Hybrid silica nanoparticles for multimodal imaging. Angew Chem Int Ed 46:3680–3682. doi:10.1002/anie.200604738
99. Lee JH, Yw J, Yeon SI, Shin JS, Cheon J (2006) Dual-mode nanoparticle probes for high-performance magnetic resonance and fluorescence imaging of neuroblastoma. Angew Chem 118:8340–8342. doi:10.1002/anie.200603052
100. Bridot J-L, Faure A-C, Laurent S, Riviere C, Billotey C, Hiba B, Janier M, Josserand V, Coll J-L, Vander Elst L (2007) Hybrid gadolinium oxide nanoparticles: multimodal contrast agents for in vivo imaging. J Am Chem Soc 129:5076–5084. doi:10.1021/ja068356j

101. Santra S, Bagwe RP, Dutta D, Stanley JT, Walter GA, Tan W, Moudgil BM, Mericle RA (2005) Synthesis and characterization of fluorescent, radio-opaque, and paramagnetic silica nanoparticles for multimodal bioimaging applications. Adv Mater 17:2165–2169. doi:10.1002/adma.200500018
102. Kim J, Lee JE, Lee J, Jang Y, Kim SW, An K, Yu JH, Hyeon T (2006) Generalized fabrication of multifunctional nanoparticle assemblies on silica spheres. Angew Chem 118:4907–4911. doi:10.1002/anie.200504107
103. Medarova Z, Pham W, Farrar C, Petkova V, Moore A (2007) In vivo imaging of siRNA delivery and silencing in tumors. Nat Med 13:372–377. doi:10.1038/nm1486
104. Nasongkla N, Bey E, Ren J, Ai H, Khemtong C, Guthi JS, Chin S-F, Sherry AD, Boothman DA, Gao J (2006) Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. Nano Lett 6:2427–2430. doi:10.1021/nl061412u
105. Pourtau L, Oliveira H, Thevenot J, Wan Y, Brisson AR, Sandre O, Miraux S, Thiaudiere E, Lecommandoux S (2013) Antibody-functionalized magnetic polymersomes: in vivo targeting and imaging of bone metastases using high resolution MRI. Adv Healthc Mater 2:1420–1424. doi:10.1002/adhm.201300061
106. Yang J, Lee CH, Ko HJ, Suh JS, Yoon HG, Lee K, Huh YM, Haam S (2007) Multifunctional magneto-polymeric nanohybrids for targeted detection and synergistic therapeutic effects on breast cancer. Angew Chem Int Ed 46:8836–8839. doi:10.1002/anie.200703554
107. Kim J, Lee JE, Lee SH, Yu JH, Lee JH, Park TG, Hyeon T (2008) Designed fabrication of a multifunctional polymer nanomedical platform for simultaneous cancer-targeted imaging and magnetically guided drug delivery. Adv Mater 20:478–483. doi:10.1002/adma.200701726
108. Kim K, Kim JH, Park H, Kim Y-S, Park K, Nam H, Lee S, Park JH, Park R-W, Kim I-S (2010) Tumor-homing multifunctional nanoparticles for cancer theragnosis: simultaneous diagnosis, drug delivery, and therapeutic monitoring. J Control Release 146:219–227. doi:10.1016/j.jconrel.2010.04.004
109. van Vlerken LE, Duan Z, Little SR, Seiden MV, Amiji MM (2008) Biodistribution and pharmacokinetic analysis of paclitaxel and ceramide administered in multifunctional polymer-blend nanoparticles in drug resistant breast cancer model. Mol Pharm 5:516–526. doi:10.1021/mp800030k
110. Park H, Yang J, Lee J, Haam S, Choi I-H, Yoo K-H (2009) Multifunctional nanoparticles for combined doxorubicin and photothermal treatments. ACS Nano 3:2919–2926. doi:10.1021/nn900215k
111. Cheng J, Teply BA, Sherifi I, Sung J, Luther G, Gu FX, Levy-Nissenbaum E, Radovic-Moreno AF, Langer R, Farokhzad OC (2007) Formulation of functionalized PLGA–PEG nanoparticles for in vivo targeted drug delivery. Biomaterials 28:869–876. doi:10.1016/j.biomaterials.2006.09.047
112. Sun B, Ranganathan B, Feng S-S (2008) Multifunctional poly (d, l-lactide-co-glycolide)/montmorillonite (PLGA/MMT) nanoparticles decorated by Trastuzumab for targeted chemotherapy of breast cancer. Biomaterials 29:475–486. doi:10.1016/j.biomaterials.2007.09.038
113. Sun B, Feng S-S (2009) Trastuzumab-functionalized nanoparticles of biodegradable copolymers for targeted delivery of docetaxel. Nanomedicine (Lond) 4:431–445. doi:10.2217/nnm.09.17
114. Danhier F, Vroman B, Lecouturier N, Crokart N, Pourcelle V, Freichels H, Jérôme C, Marchand-Brynaert J, Feron O, Préat V (2009) Targeting of tumor endothelium by RGD-grafted PLGA-nanoparticles loaded with paclitaxel. J Control Release 140:166–173. doi:10.1016/j.jconrel.2009.08.011
115. Patil YB, Toti US, Khdair A, Ma L, Panyam J (2009) Single-step surface functionalization of polymeric nanoparticles for targeted drug delivery. Biomaterials 30:859–866. doi:10.1016/j.biomaterials.2008.09.056
116. Shi D, Cho HS, Chen Y, Xu H, Gu H, Lian J, Wang W, Liu G, Huth C, Wang L (2009) Fluorescent polystyrene–Fe3O4 composite nanospheres for in vivo imaging and hyperthermia. Adv Mater 21:2170–2173. doi:10.1002/adma.200803159

117. Schleich N, Sibret P, Danhier P, Ucakar B, Laurent S, Muller R, Jérôme C, Gallez B, Préat V, Danhier F (2013) Dual anticancer drug/superparamagnetic iron oxide-loaded PLGA-based nanoparticles for cancer therapy and magnetic resonance imaging. Int J Pharm 447:94–101. doi:10.1016/j.ijpharm.2013.02.042
118. Kopelman R, Lee Koo Y-E, Philbert M, Moffat BA, Ramachandra Reddy G, McConville P, Hall DE, Chenevert TL, Bhojani MS, Buck SM (2005) Multifunctional nanoparticle platforms for in vivo MRI enhancement and photodynamic therapy of a rat brain cancer. J Magn Magn Mater 293:404–410. doi:10.1016/j.jmmm.2005.02.061
119. Reddy GR, Bhojani MS, McConville P, Moody J, Moffat BA, Hall DE, Kim G, Koo Y-EL, Woolliscroft MJ, Sugai JV (2006) Vascular targeted nanoparticles for imaging and treatment of brain tumors. Clin Cancer Res 12:6677–6686. doi:10.1158/1078-0432.CCR-06-0946
120. Chatterjee DK, Zhang Y (2007) Multi-functional nanoparticles for cancer therapy. Sci Technol Adv Mater 8:131–133. doi:10.1016/j.stam.2006.09.008
121. Pan J, Liu Y, Feng S-S (2010) Multifunctional nanoparticles of biodegradable copolymer blend for cancer diagnosis and treatment. Nanomedicine 5:347–360. doi:10.2217/nnm.10.13
122. Yang X, Grailer JJ, Rowland IJ, Javadi A, Hurley SA, Steeber DA, Gong S (2010) Multifunctional SPIO/DOX-loaded wormlike polymer vesicles for cancer therapy and MR imaging. Biomaterials 31:9065–9073. doi:10.1016/j.biomaterials.2010.08.039
123. Maeng JH, Lee D-H, Jung KH, Bae Y-H, Park I-S, Jeong S, Jeon Y-S, Shim C-K, Kim W, Kim J (2010) Multifunctional doxorubicin loaded superparamagnetic iron oxide nanoparticles for chemotherapy and magnetic resonance imaging in liver cancer. Biomaterials 31:4995–5006. doi:10.1016/j.biomaterials.2010.02.068
124. Ling Y, Wei K, Luo Y, Gao X, Zhong S (2011) Dual docetaxel/superparamagnetic iron oxide loaded nanoparticles for both targeting magnetic resonance imaging and cancer therapy. Biomaterials 32:7139–7150. doi:10.1016/j.biomaterials.2011.05.089
125. Cheng F-Y, Su C-H, Wu P-C, Yeh C-S (2010) Multifunctional polymeric nanoparticles for combined chemotherapeutic and near-infrared photothermal cancer therapy in vitro and in vivo. Chem Commun 46:3167–3169. doi:10.1039/b919172k
126. Cho H-S, Dong Z, Pauletti GM, Zhang J, Xu H, Gu H, Wang L, Ewing RC, Huth C, Wang F (2010) Fluorescent, superparamagnetic nanospheres for drug storage, targeting, and imaging: a multifunctional nanocarrier system for cancer diagnosis and treatment. ACS Nano 4:5398–5404. doi:10.1021/nn101000e
127. Guo R, Zhang L, Qian H, Li R, Jiang X, Liu B (2010) Multifunctional nanocarriers for cell imaging, drug delivery, and near-IR photothermal therapy. Langmuir 26:5428–5434. doi:10.1021/la903893n
128. Tuszynski MH, Thal L, Pay M, Salmon DP, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 11:551–555. doi:10.1038/nm1239
129. Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, Bland RJ, Young D, Strybing K, Eidelberg D (2007) Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. Lancet 369:2097–2105. doi:10.1016/S0140-6736(07)60982-9
130. Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit J-P (2008) Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials 29:3477–3496. doi:10.1016/j.biomaterials.2008.04.036
131. Reischl D, Zimmer A (2009) Drug delivery of siRNA therapeutics: potentials and limits of nanosystems. Nanomed Nanotechnol Biol Med 5:8–20. doi:10.1016/j.nano.2008.06.001
132. Roy I, Stachowiak MK, Bergey EJ (2008) Nonviral gene transfection nanoparticles: function and applications in the brain. Nanomed Nanotechnol Biol Med 4:89–97. doi:10.1016/j.nano.2008.01.002
133. Jain S, Kumar S, Agrawal AK, Thanki K, Banerjee UC (2013) Enhanced transfection efficiency and reduced cytotoxicity of novel lipid–polymer hybrid nanoplexes. Mol Pharm 10:2416–2425. doi:10.1021/mp400036w

134. Glover DJ, Lipps HJ, Jans DA (2005) Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet 6:299–310. doi:10.1038/nrg1577
135. Schmidt-Wolf GD, Schmidt-Wolf IG (2003) Non-viral and hybrid vectors in human gene therapy: an update. Trends Mol Med 9:67–72. doi:10.1016/S1471-4914(03)00005-4
136. Pack DW, Hoffman AS, Pun S, Stayton PS (2005) Design and development of polymers for gene delivery. Nat Rev Drug Discov 4:581–593. doi:10.1038/nrd1775
137. Lin E-H, Chang H-Y, Yeh S-D, Yang K-Y, Hu H-S, Wu C-W (2013) Polyethyleneimine and DNA nanoparticles-based gene therapy for acute lung injury. Nanomed Nanotechnol Biol Med 9:1293–1303. doi:10.1016/j.nano.2013.05.004
138. Vijayanathan V, Thomas T, Thomas TJ (2002) DNA nanoparticles and development of DNA delivery vehicles for gene therapy. Biochemistry 41:14085–14094. doi:10.1021/bi0203987
139. Kim HO, Kim E, An Y, Choi J, Jang E, Choi EB, Kukreja A, Kim MH, Kang B, Kim DJ (2013) A biodegradable polymersome containing Bcl-xL siRNA and doxorubicin as a dual delivery vehicle for a synergistic anticancer effect. Macromol Biosci 13:745–754. doi:10.1002/mabi.201200448
140. Kim Y, Tewari M, Pajerowski JD, Cai S, Sen S, Williams J, Sirsi S, Lutz G, Discher DE (2009) Polymersome delivery of siRNA and antisense oligonucleotides. J Control Release 134:132–140. doi:10.1016/j.jconrel.2008.10.020
141. Kommareddy S, Amiji M (2005) Preparation and evaluation of thiol-modified gelatin nanoparticles for intracellular DNA delivery in response to glutathione. Bioconjug Chem 16:1423–1432. doi:10.1021/bc050146t
142. Kommareddy S, Amiji M (2007) Antiangiogenic gene therapy with systemically administered sFlt-1 plasmid DNA in engineered gelatin-based nanovectors. Cancer Gene Ther 14:488–498. doi:10.1038/sj.cgt.7701041
143. Pangburn TO, Georgiou K, Bates FS, Kokkoli E (2012) Targeted polymersome delivery of siRNA induces cell death of breast cancer cells dependent upon Orai3 protein expression. Langmuir 28:12816–12830. doi:10.1021/la300874z
144. Bhavsar MD, Amiji MM (2007) Gastrointestinal distribution and in vivo gene transfection studies with nanoparticles-in-microsphere oral system (NiMOS). J Control Release 119:339–348. doi:10.1016/j.jconrel.2007.03.006
145. Zhang J, Sun H, Ma PX (2010) Host– guest interaction mediated polymeric assemblies: multifunctional nanoparticles for drug and gene delivery. ACS Nano 4:1049–1059. doi:10.1021/nn901213a
146. Davis ME (2009) The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. Mol Pharm 6:659–668. doi:10.1021/mp900015y
147. Bellocq NC, Pun SH, Jensen GS, Davis ME (2003) Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. Bioconjug Chem 14:1122–1132. doi:10.1021/bc034125f
148. Khalil I, Kogure K, Futaki S, Hama S, Akita H, Ueno M, Kishida H, Kudoh M, Mishina Y, Kataoka K (2007) Octaarginine-modified multifunctional envelope-type nanoparticles for gene delivery. Gene Ther 14:682–689. doi:10.1038/sj.gt.3302910
149. Kogure K, Akita H, Harashima H (2007) Multifunctional envelope-type nano device for non-viral gene delivery: concept and application of Programmed Packaging. J Control Release 122:246–251. doi:10.1016/j.jconrel.2007.06.018
150. Kogure K, Akita H, Yamada Y, Harashima H (2008) Multifunctional envelope-type nano device (MEND) as a non-viral gene delivery system. Adv Drug Deliv Rev 60:559–571. doi:10.1016/j.addr.2007.10.007
151. Hatakeyama H, Akita H, Harashima H (2011) A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. Adv Drug Deliv Rev 63:152–160. doi:10.1016/j.addr.2010.09.001
152. Nakamura Y, Kogure K, Futaki S, Harashima H (2007) Octaarginine-modified multifunctional envelope-type nano device for siRNA. J Control Release 119:360–367. doi:10.1016/j.jconrel.2007.03.010

153. Hatakeyama H, Ito E, Akita H, Oishi M, Nagasaki Y, Futaki S, Harashima H (2009) A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles in vitro and in vivo. J Control Release 139:127–132. doi:10.1016/j.jconrel.2009.06.008
154. El-Sayed A, Futaki S, Harashima H (2009) Delivery of macromolecules using arginine-rich cell-penetrating peptides: ways to overcome endosomal entrapment. AAPS J 11:13–22. doi:10.1208/s12248-008-9071-2
155. Khalil IA, Hayashi Y, Mizuno R, Harashima H (2011) Octaarginine-and pH sensitive fusogenic peptide-modified nanoparticles for liver gene delivery. J Control Release 156:374–380. doi:10.1016/j.jconrel.2011.08.012
156. Akita H, Kogure K, Moriguchi R, Nakamura Y, Higashi T, Nakamura T, Serada S, Fujimoto M, Naka T, Futaki S (2010) Nanoparticles for ex vivo siRNA delivery to dendritic cells for cancer vaccines: Programmed endosomal escape and dissociation. J Control Release 143:311–317. doi:10.1016/j.jconrel.2010.01.012
157. Guo S, Qiao Y, Wang W, He H, Deng L, Xing J, Xu J, Liang X-J, Dong A (2010) Poly (ε-caprolactone)-graft-poly (2-(N, N-dimethylamino) ethyl methacrylate) nanoparticles: pH dependent thermo-sensitive multifunctional carriers for gene and drug delivery. J Mater Chem 20:6935–6941. doi:10.1039/C0JM00506A
158. Nishikawa M, Yamauchi M, Morimoto K, Ishida E, Takakura Y, Hashida M (2000) Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. Gene Ther 7:548–555. doi:10.1038/sj.gt.3301140
159. Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, Molema G, Lu PY, Scaria PV, Woodle MC (2004) Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res 32:e149–e149. doi:10.1093/nar/gnh140
160. Suk JS, Suh J, Choy K, Lai SK, Fu J, Hanes J (2006) Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. Biomaterials 27:5143–5150. doi:10.1016/j.biomaterials.2006.05.013
161. Magadala P, Amiji M (2008) Epidermal growth factor receptor-targeted gelatin-based engineered nanocarriers for DNA delivery and transfection in human pancreatic cancer cells. AAPS J 10:565–576. doi:10.1208/s12248-008-9065-0
162. Saeed AO, Magnusson JP, Moradi E, Soliman M, Wang W, Stolnik S, Thurecht KJ, Howdle SM, Alexander C (2011) Modular construction of multifunctional bioresponsive cell-targeted nanoparticles for gene delivery. Bioconjug Chem 22:156–168. doi:10.1021/bc100149g
163. Patil YB, Swaminathan SK, Sadhukha T, Ma L, Panyam J (2010) The use of nanoparticle-mediated targeted gene silencing and drug delivery to overcome tumor drug resistance. Biomaterials 31:358–365. doi:10.1016/j.biomaterials.2009.09.048
164. Chen G, Chen W, Wu Z, Yuan R, Li H, Gao J, Shuai X (2009) MRI-visible polymeric vector bearing CD3 single chain antibody for gene delivery to T cells for immunosuppression. Biomaterials 30:1962–1970. doi:10.1016/j.biomaterials.2008.12.043
165. Juillerat-Jeanneret L (2008) The targeted delivery of cancer drugs across the blood–brain barrier: chemical modifications of drugs or drug-nanoparticles? Drug Discov Today 13:1099–1106. doi:10.1016/j.drudis.2008.09.005
166. Wong HL, Wu XY, Bendayan R (2012) Nanotechnological advances for the delivery of CNS therapeutics. Adv Drug Deliv Rev 64:686–700. doi:10.1016/j.addr.2011.10.007
167. Kreuter J, Shamenkov D, Petrov V, Ramge P, Cychutek K, Koch-Brandt C, Alyautdin R (2002) Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood-brain barrier. J Drug Target 10:317–325. doi:10.1080/10611860290031877
168. Kreuter J, Alyautdin RN, Kharkevich DA, Ivanov AA (1995) Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles). Brain Res 674:171–174. doi:10.1016/0006-8993(95)00023-J
169. Gulyaev AE, Gelperina SE, Skidan IN, Antropov AS, Kivman GY, Kreuter J (1999) Significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles. Pharm Res 16:1564–1569. doi:10.1023/A:1018983904537

170. Kreuter J (2012) Nanoparticulate systems for brain delivery of drugs. Adv Drug Deliv Rev 64:213–222. doi:10.1016/S0169-409X(00)00122-8
171. Alyautdin R, Tezikov E, Ramge P, Kharkevich D, Begley D, Kreuter J (1998) Significant entry of tubocurarine into the brain of rats by adsorption to polysorbate 80-coated polybutylcyanoacrylate nanoparticles: an in situ brain perfusion study. J Microencapsul 15:67–74. doi:10.3109/02652049809006836
172. Alyautdin RN, Petrov VE, Langer K, Berthold A, Kharkevich DA, Kreuter J (1997) Delivery of loperamide across the blood-brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. Pharm Res 14:325–328. doi:10.1023/A:1012098005098
173. Gelperina S, Smirnova Z, Khalanskiy A, Skidan I, Bobruskin A, Kreuter J. (2000) Chemotherapy of brain tumours using doxorubicin bound to polysorbate 80-coated nanoparticles. In: Proceedings of the 3rd World Meeting APV/APGI, Berlin, 2000, pp. 441–442
174. Kreuter J, Petrov V, Kharkevich D, Alyautdin R (1997) Influence of the type of surfactant on the analgesic effects induced by the peptide dalargin after its delivery across the blood–brain barrier using surfactant-coated nanoparticles. J Control Release 49:81–87. doi:10.1016/S0168-3659(97)00061-8
175. Schröder U, Sabel BA (1996) Nanoparticles, a drug carrier system to pass the blood-brain barrier, permit central analgesic effects of iv dalargin injections. Brain Res 710:121–124. doi:10.1016/0006-8993(95)01375-X
176. Schroeder U, Sommerfeld P, Sabel BA (1998) Efficacy of oral dalargin-loaded nanoparticle delivery across the blood–brain barrier. Peptides 19:777–780. doi:10.1016/S0196-9781(97)00474-9
177. Schroeder U, Sommerfeld P, Ulrich S, Sabel BA (1998) Nanoparticle technology for delivery of drugs across the blood–brain barrier. J Pharm Sci 87:1305–1307. doi:10.1021/js980084y
178. Chen Y, Liu L (2012) Modern methods for delivery of drugs across the blood–brain barrier. Adv Drug Deliv Rev 64:640–665. doi:10.1016/j.addr.2011.11.010
179. Hans M, Lowman A (2002) Biodegradable nanoparticles for drug delivery and targeting. Curr Opin Solid State Mater Sci 6:319–327. doi:10.1016/S1359-0286(02)00117-1
180. Wilson B, Samanta MK, Santhi K, Kumar KPS, Paramakrishnan N, Suresh B (2008) Poly (n-butylcyanoacrylate) nanoparticles coated with polysorbate 80 for the targeted delivery of rivastigmine into the brain to treat Alzheimer's disease. Brain Res 1200:159–168. doi:10.1016/j.brainres.2008.01.039
181. Liu G, Garrett MR, Men P, Zhu X, Perry G, Smith MA (2005) Nanoparticle and other metal chelation therapeutics in Alzheimer disease. BBA-Mol Basis Dis 1741:246–252. doi:10.1016/j.bbadis.2005.06.006
182. Christen Y (2000) Oxidative stress and Alzheimer disease. Am J Clin Nutr 71:621–629
183. Hazel JR, Eugene Williams E (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog Lipid Res 29:167–227
184. Henderson BW, Dougherty TJ (1992) How does photodynamic therapy work? Photochem Photobiol 55:145–157. doi:10.1111/j.1751-1097.1992.tb04222.x
185. Tang W, Xu H, Kopelman R, Philbert MA (2005) Photodynamic characterization and in vitro application of methylene blue-containing nanoparticle platforms. Photochem Photobiol 81:242–249. doi:10.1111/j.1751-1097.2005.tb00181.x
186. Porkka K, Laakkonen P, Hoffman JA, Bernasconi M, Ruoslahti E (2002) A fragment of the HMGN2 protein homes to the nuclei of tumor cells and tumor endothelial cells in vivo. Proc Natl Acad Sci U S A 99:7444–7449. doi:10.1073/pnas.062189599
187. Abels C (2004) Targeting of the vascular system of solid tumours by photodynamic therapy (PDT). Photochem Photobiol Sci 3:765–771. doi:10.1039/b314241h
188. Tian X-H, Lin X-N, Wei F, Feng W, Huang Z-C, Wang P, Ren L, Diao Y (2011) Enhanced brain targeting of temozolomide in polysorbate-80 coated polybutylcyanoacrylate nanoparticles. Int J Nanomedicine 6:445–452. doi:10.2147/IJN.S16570
189. Kuo Y-C, Chen H-H (2006) Effect of nanoparticulate polybutylcyanoacrylate and methylmethacrylate–sulfopropylmethacrylate on the permeability of zidovudine and lamivudine

across the in vitro blood–brain barrier. Int J Pharm 327:160–169. doi:10.1016/j.ijpharm.2006.07.044
190. Roney C, Kulkarni P, Arora V, Antich P, Bonte F, Wu A, Mallikarjuana NN, Manohar S, Liang H-F, Kulkarni AR, Sung H-W, Sairam M, Aminabhavi TM (2005) Targeted nanoparticles for drug delivery through the blood–brain barrier for Alzheimer's disease. J Control Release 108:193–214. doi:10.1016/j.jconrel.2005.07.024
191. Calvo P, Gouritin B, Chacun H, Desmaële D, D'Angelo J, Noel J-P, Georgin D, Fattal E, Andreux JP, Couvreur P (2001) Long-circulating PEGylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery. Pharm Res 18:1157–1166. doi:10.1023/A:1010931127745
192. Gao X, Wu B, Zhang Q, Chen J, Zhu J, Zhang W, Rong Z, Chen H, Jiang X (2007) Brain delivery of vasoactive intestinal peptide enhanced with the nanoparticles conjugated with wheat germ agglutinin following intranasal administration. J Control Release 121:156–167. doi:10.1016/j.jconrel.2007.05.026
193. Ulbrich K, Hekmatara T, Herbert E, Kreuter J (2009) Transferrin-and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood–brain barrier (BBB). Eur J Pharm Biopharm 71:251–256. doi:10.1016/j.ejpb.2008.08.021
194. Blumberg BS, London WT (1981) Hepatitis B virus and the prevention of primary hepatocellular carcinoma. N Engl J Med 304:782–784. doi:10.1056/NEJM198103263041312
195. Mimi CY, Mack T, Hanisch R, Peters RL, Henderson BE, Pike MC (1983) Hepatitis, alcohol consumption, cigarette smoking, and hepatocellular carcinoma in Los Angeles. Cancer Res 43:6077–6079
196. Ohnishi K, Iida S, Iwama S, Goto N, Nomura F, Takashi M, Mishima A, Kono K, Kimura K, Musha H (1982) The effect of chronic habitual alcohol intake on the development of liver cirrhosis and hepatocellular carcinoma: relation to hepatitis B surface antigen carriage. Cancer49:672–677. doi:10.1002/1097-0142(19820215)49:4<672::AID-CNCR2820490415>3.0.CO;2-#
197. Ashwell G, Harford J (1982) Carbohydrate-specific receptors of the liver. Annu Rev Biochem 51:531–554. doi:10.1146/annurev.bi.51.070182.002531
198. Wu J, Nantz MH, Zern MA (2002) Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications. Front Biosci 7:d717
199. Ciechanover A, Schwartz AL, Lodish HF (1983) Sorting and recycling of cell surface receptors and endocytosed ligands: the asialoglycoprotein and transferrin receptors. J Cell Biochem 23:107–130. doi:10.1002/jcb.240230111
200. Liang H-F, Chen C-T, Chen S-C, Kulkarni AR, Chiu Y-L, Chen M-C, Sung H-W (2006) Paclitaxel-loaded poly (γ-glutamic acid)-poly (lactide) nanoparticles as a targeted drug delivery system for the treatment of liver cancer. Biomaterials 27:2051–2059. doi:10.1016/j.biomaterials.2005.10.027
201. Cho C, Cho K, Park I, Kim S, Sasagawa T, Uchiyama M, Akaike T (2001) Receptor-mediated delivery of all trans-retinoic acid to hepatocyte using poly (L-lactic acid) nanoparticles coated with galactose-carrying polystyrene. J Control Release 77:7–15. doi:10.1016/S0168-3659(01)00390-X
202. Jiang H-L, Kwon J-T, Kim E-M, Kim Y-K, Arote R, Jere D, Jeong H-J, Jang M-K, Nah J-W, Xu C-X (2008) Galactosylated poly (ethylene glycol)-chitosan-graft-polyethylenimine as a gene carrier for hepatocyte-targeting. J Control Release 131:150–157. doi:10.1016/j.jconrel.2008.07.029
203. Wu D-Q, Lu B, Chang C, Chen C-S, Wang T, Zhang Y-Y, Cheng S-X, Jiang X-J, Zhang X-Z, Zhuo R-X (2009) Galactosylated fluorescent labeled micelles as a liver targeting drug carrier. Biomaterials 30:1363–1371. doi:10.1016/j.biomaterials.2008.11.027
204. Sawamura T, Nakada H, Hazama H, Shiozaki Y, Sameshima Y, Tashiro Y (1984) Hyperasialoglycoproteinemia in patients with chronic liver diseases and/or liver cell carcinoma. Asialoglycoprotein receptor in cirrhosis and liver cell carcinoma. Gastroenterology 87:1217–1221

205. Tian Q, Zhang C-N, Wang X-H, Wang W, Huang W, Cha R-T, Wang C-H, Yuan Z, Liu M, Wan H-Y (2010) Glycyrrhetinic acid-modified chitosan/poly (ethylene glycol) nanoparticles for liver-targeted delivery. Biomaterials 31:4748–4756. doi:10.1016/j.biomaterials.2010.02.042
206. Asl MN, Hosseinzadeh H (2008) Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. Phytother Res 22:709–724
207. Ishida S, Sakiya Y, Ichikawa T, Taira Z (1993) Uptake of glycyrrhizin by isolated rat hepatocytes. Biol Pharm Bull 16:293–297
208. Ishida S, Sakiya Y, Taira Z (1994) Disposition of glycyrrhizin in the perfused liver of rats. Biol Pharm Bull 17:960–969
209. Negishi M, Irie A, Nagata N, Ichikawa A (1991) Specific binding of glycyrrhetinic acid to the rat liver membrane. Biochim Biophys Acta 1066:77–82. doi:10.1016/0005-2736(91)90253-5
210. Lin A, Liu Y, Huang Y, Sun J, Wu Z, Zhang X, Ping Q (2008) Glycyrrhizin surface-modified chitosan nanoparticles for hepatocyte-targeted delivery. Int J Pharm 359:247–253. doi:10.1016/j.ijpharm.2008.03.039
211. Agarwal A, Saraf S, Asthana A, Gupta U, Gajbhiye V, Jain NK (2008) Ligand based dendritic systems for tumor targeting. Int J Pharm 350:3–13. doi:10.1016/j.ijpharm.2007.09.024
212. Zhang L, Gong F, Zhang F, Ma J, Zhang P, Shen J (2013) Targeted therapy for human hepatic carcinoma cells using folate-functionalized polymeric micelles loaded with superparamagnetic iron oxide and sorafenib in vitro. Int J Nanomedicine 8:1517–1524. doi:10.2147/IJN.S43263
213. Kim TH, Park IK, Nah JW, Choi YJ, Cho CS (2004) Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier. Biomaterials 25:3783–3792. doi:10.1016/j.biomaterials.2003.10.063
214. Mansouri S, Cuie Y, Winnik F, Shi Q, Lavigne P, Benderdour M, Beaumont E, Fernandes JC (2006) Characterization of folate-chitosan-DNA nanoparticles for gene therapy. Biomaterials 27:2060–2065. doi:10.1016/j.biomaterials.2005.09.020
215. Liu Y, Chen Z, Liu C, Yu D, Lu Z, Zhang N (2011) Gadolinium-loaded polymeric nanoparticles modified with Anti-VEGF as multifunctional MRI contrast agents for the diagnosis of liver cancer. Biomaterials 32:5167–5176. doi:10.1016/j.biomaterials.2011.03.077
216. Hong G, Yuan R, Liang B, Shen J, Yang X, Shuai X (2008) Folate-functionalized polymeric micelle as hepatic carcinoma-targeted, MRI-ultrasensitive delivery system of antitumor drugs. Biomed Microdevices 10:693–700. doi:10.1007/s10544-008-9180-9

Chapter 16
Polymeric Micelles in Targeted Drug Delivery

Rayasa S. Ramachandra Murthy

Abbreviations

AFM	Atomic force microscopy
ATRA	All-*trans* retinoic acid
AUC	Area under the curve
CMC	Critical micellar concentration
CMT	Critical micellization temperature
CPT	Camptothecin
CsA	Cyclosporine A
DNA	Deoxy ribonucleic acid
DOX	Doxorubicin
DSPE	Distearoyl phosphatidyl ethanolamine
EPR	Enhanced permeability and retention
F-5-CADA	Fluorescein-5-carbonyl azide diacetate
FA	Folic acid
Gd	Gadolinium
HEMAm	*N*-(2-hydroxyethyl) methacrylamide
HLB	Hydrophilic–lipophilic balance
LCST	Low critical solution temperature
MHC	Minimal hydrotrope concentration
MRI	Magnetic resonance imaging
PAsp	Poly(aspartic acid)

R.S.R. Murthy (✉)
Faculty of Technology & Engineering, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India

103, Sumukha Green Ville, Sarvabhauma Nagara, Bilekahalli, Banner ghatta Road, IIMB Post office, Bangalore 560076, India
e-mail: m_gaya@rediffmail.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_16

PBLA	Poly(benzyl-L-aspartate)
PCL	Poly(e-caprolactone)
PDLLA	PEG-b-poly(D,L-lactic acid)
PEG	Poly(ethylene glycol)
PEO	Polyethylene oxide
PET	Positron emission tomography
P-gp	*P*-glycoprotein
PICMs	Polyion complex micelles
PM	Polymeric micelle
PMMA	Poly(methacrylate)
PPO	Poly(propylene oxide)
PTX	Paclitaxel
PVA	Poly(vinyl alcohol)
RNA	Ribonucleic acid
RT	Room temperature
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

16.1 Introduction

Amphiphilic molecules or surfactant monomers that possess a polar head and a lipophilic tail could show changes in their physicochemical properties in solutions. These changes are associated with the orientation and association of amphiphilic molecules in solution resulting in the formation of structures called micelles. The micelles internally have a hydrophobic core and externally a hydrophilic surface. Micelles are generally made up of 50–200 monomers (an average number of monomers forming micelle at any given time is termed as the aggregation number). The radius of a spherical micelle is 1–3 nm, and thus they lie in the colloidal range [1, 2]. The major driving force behind self-association of amphiphilic molecules is the decrease of free energy of the system. The decrease in free energy is a result of removal of hydrophobic fragments from the aqueous surroundings with the formation of a micelle core stabilized with hydrophilic fragments exposed into water. The factors affecting the process of micelle formation are the size of the hydrophobic domain in the amphiphilic molecule, concentration of amphiphiles, temperature, and solvent. The minimum concentration of amphiphilic molecules to form assembly is called critical micelle concentration (CMC). At low concentrations in medium, these amphiphilic molecules exist separately, and are so small that they appear to be subcolloidal. Below the CMC, the amphiphile undergo adsorption at the air–water interface. As the total concentration of the amphiphile is increased up to CMC, the interface as well as the bulk phase is saturated with monomers. Any further amphiphile added in excess of CMC results in the aggregation of monomers in the bulk phase, such that the free energy of the system is reduced. The temperature below which amphiphilic molecules exist as unimers and above which as aggregates is the critical micellization temperature (CMT) [1, 3, 4].

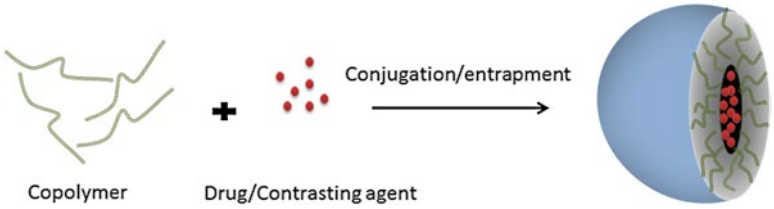

Fig. 16.1 Design of a polymeric micelle carrier system

16.2 Polymeric Micelle

A polymeric micelle is a macromolecular assembly that forms from synthetic amphiphilic block copolymers or graft copolymers. It has a spherical inner core and an outer shell [5]. As shown in Fig.16.1, which features an AB-type block copolymer, a micellar structure forms in an aqueous medium if one segment of the block copolymer can provide interchain cohesive interactions sufficient for the micelle formation.

Here, Amphiphilic block or graft copolymers behave in the same manner as that of conventional amphiphiles and in aqueous solution, above CMC, these polymers form polymeric micelles. In contrast to the micelles of conventional surfactant monomers, in polymeric micelles there is a covalent linkage in individual surfactant molecules within the hydrophobic core. This linkage prevents dynamic exchange of monomers between free solution and the micellar pseudo-phase which confers rigidity and stability to the polymeric micelles [6]. The aggregation number of polymeric micelles is of the magnitude of several hundreds and the diameter ranges from 10 to 100 nm. Factors controlling the size of the polymeric micelles include molecular weight of the amphiphilic block copolymer, aggregation number of the amphiphiles, relative proportion of hydrophilic and hydrophobic chains, and the preparation process [7]. In aqueous medium amphiphilic block copolymers can principally self-assemble into spherical micelles, worm-like or cylindrical micelles, and polymer vesicles or polymersomes. Main factor governing the morphology of micelles is the hydrophilic–hydrophobic balance of the block copolymer defined by the hydrophilic volume fraction, f. For amphiphilic block copolymers with value of nearly 35 %, polymer vesicles are formed, whereas, for f value more than 45 %, spherical micelles are formed from self-assembly [8, 9]. By using amphiphiles of more complicated molecular design, e.g., miktoarm star copolymers, or by varying the experimental conditions for self-assembly more complex morphologies such as that of crew-cut micelles, multicompartment micelles, toroids, etc. may be obtained [10].

16.3 Types of Polymeric Micelles

On the basis of the type of intermolecular forces governing the segregation of the core segment from the aqueous environment, polymeric micelles can be classified in three main categories, i.e., micelles formed by hydrophobic interactions, those resulting from electrostatic interactions (polyion complex micelles), and micelles from metal complexation.

16.3.1 Conventional

These micelles are formed by hydrophobic interactions between the core segment and the corona region of amphiphilic block copolymer in the aqueous environment. One of the simplest example of this type is the micelle formed by hydrophobic interactions in poly(ethylene oxide)-b-poly(propylene oxide)-bpoly(ethylene oxide) [11].

16.3.2 Polyion Complex Micelles

Polymeric micelles are also formed by electrostatic interactions between two oppositely charged moieties, such as polyelectrolytes and these micelles are termed polyion complex micelles (PICMs). When oppositely charged polymers are added in the solution, they can penetrate in the corona of the micelle and give rise to polyionic micelle. The electrostatic forces and the van der Waals force of interaction control the structure and size of the charged micelle coronas. PICMs have some peculiar features such as simple synthetic route, easy self-assembly in aqueous medium, structural stability, high drug loading capacity, and prolonged circulation in the blood. The preparation of micelles being carried out in aqueous medium without involvement of any organic solvents, they overcome the associated side-effects produced by the residual organic solvents. The core of the PICMs can entrap many therapeutic agents such as hydrophobic compounds, hydrophilic compounds, metal complexes, and charged macromolecules through electrostatic, hydrophobic, hydrogen bonding interactions and release them after receiving a suitable trigger. Because of these reasons, the PICMs have a great potential for drug release, especially for the delivery of charged drugs, antisense oligonucleotides, DNA, and enzymes [12, 13]. Recently, Jung et al. prepared polymeric micelles of methoxypoly(ethylene glycol)-grafted-chitosan encapsulating all trans retinoic acid through the formation of a polyion complex between the amine group of chitosan and the carboxylic acid group of all-*trans* retinoic acid. The PICMs were designed for drug delivery to the brain tumor. The sizes of PICMs were about 50–200 nm and the loading efficiency of micelle was higher than 80 % (w/w).

Table 16.1 Type of amphiphilic copolymers and their possible structures

Type of copolymers	Representation of structure[a]	Example of polymers
Block copolymers	di—block AAAAAAABBBBBB	Poly(styrene)-b-poly(ethylene oxide)
	tri—block AAAABBBBBAAAA	Poly(ethylene oxide)-b-poly (propylene oxide)-b-poly(ethylene oxide)
Graft copolymers	AAAAAAAAAAAA B B B B B B B	*N*-phthaloylchitosan-g-polycaprolactone

[a]*A* hydrophilic unit, *B* hydrophobic unit

16.3.3 Non-covalently Connected Polymeric Micelles

Polymeric micelles are also prepared by a novel "block-copolymer-free" technique. Here, polymeric micelles are obtained via self-assemblage of homopolymer, random copolymer, graft copolymer or oligomer by inter polymer hydrogen bonding complexation. Core and shell are non-covalently connected at their homopolymer chain end by specific inter molecular interactions such as H-bonding or metal-ligand interactions in the resultant structures and hence these are termed as non-covalently connected micelles. Jiang et al. prepared the intermolecular complexes with poly(4-vinylpyridine) as the backbone and carboxyl terminated poly butadiene as the grafts due to hydrogen bonding using chloroform as a common solvent.

16.4 Polymers used in Polymeric Micelle

Amphiphilic copolymers, either block copolymers (di, tri, or tetra) or graft copolymers, can be used to form polymeric micelle. A graft copolymer is one which comprises a polymer chain as a backbone and another polymer chain as side "grafted" parts. These copolymers usually demonstrate properties of both the polymeric backbones as well as of the graft [14]. Table 16.1 shows different possible structures of amphiphilic copolymers with representative example of each class.

Amphiphilic diblock AB-type or triblock ABA-type copolymers with the length of a hydrophilic block exceeding to some extent that of a hydrophobic one would self-assemblage to form spherical micelles in aqueous solutions. If the length of a hydrophilic block is too large, copolymers exist in water as individual molecules (unimers), and molecules with lengthy hydrophobic blocks develop various structures. Examples of different amphiphilic copolymers that have been investigated for producing micelles are given in Table 16.2.

Table 16.2 Various polymers used to prepare polymeric micelle

S. No.	Polymer	Reference
1.	*N*-phthaloylcarboxymethylchitosan	[15]
2.	Poly(2-ethylhexyl acrylate)-b-poly(acrylic acid)	[16]
3.	Poly(tert-butyl acrylate)-b-poly(2-vinylpyridine)	[17]
4.	Poly(ethylene oxide)-b-polycaprolactone	[18]
5.	Poly(e-caprolactone)-b-poly(ethylene glycol)-b-poly(e-caprolactone)	[19, 20]
6.	Poly(e-caprolactone)-b-poly(methacrylic acid)	[21]
7.	Poly(ethyleneglycol)-b-poly(e-caprolactone-co-trimethylenecarbonate)	[22]
8.	Poly(aspartic acid)-b-polylactide	[23]
9.	Poly(ethylene glycol)-block-poly(aspartate-hydrazide)	[24]
10.	Poly(*N*-isopropyl acrylamide-co-methacryl acid)-g-poly(D,L-lactide)	[25]
11.	Stearic acid-grafted chitosan oligosaccharide	[26]

16.5 Preparation of Polymeric Micelle

Polymeric micelles are prepared by either of the two following methods.

1. By direct dissolution: This method is used for block copolymers with low molecular weight and short length of the insoluble block. Here, micelles are prepared by direct dissolution in a selective solvent for one of the blocks. To facilitate dissolution, stirring, thermal, or ultrasound treatments can be used. The micelle formed gets trapped in a solvent that is a strong nonsolvent for the core and thus become stable.
2. By counter solvent method: molecularly dissolved chains of block copolymer in a selective solvent for one of the blocks is added with a non-solvent for the other block to form micelle. Alternatively, temperature or pH variations may be used for micelle formation [27].

16.6 Preparation of Drug-Loaded Micelles

Drug-loaded polymeric micelles can be prepared mainly by three common approaches: direct dissolution, solvent evaporation, and dialysis.

Direct dissolution method: In this method, the amphiphilic copolymer and drug in water is prepared at or above CMC. The copolymer and the drug self-assemble in water to form drug-loaded micelles. But this method usually is associated with low drug loading which can be enhanced by increase in temperature. Alternately a thin evaporated film of drug can be prepared before the addition of copolymer.

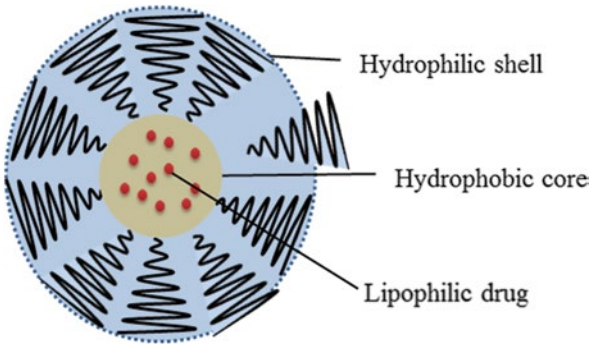

Fig. 16.2 Schematic of a hydrophobically assembled polymer micelle. The hydrophobic core loading lipophilic drugs is protected from the environment by the hydrophilic shell

Solvent evaporation or solution-casting technique: In this technique, copolymer and the drug are dissolved in a volatile organic solvent and a thin film is formed by evaporating the solvent. Drug-loaded polymeric micelles are obtained by reconstitution of film with water. When the core forming blocks are long and more hydrophobic, the two above-mentioned techniques are unsuitable.

Dialysis technique: Micelles from copolymers having long and more hydrophobic core forming blocks have the potential to solubilize large amounts of poorly water-soluble drugs. In these cases, the dialysis technique can be used to prepare drug-loaded micelles. Solutions of the drug and the polymer in organic solvent are placed in the dialysis bag, and the solvent is exchanged with water by immersing bag into water, inducing micelle assembly [28, 29]. However, emulsification involving use of chlorinated solvents is not safer and dialysis process often requires more than 36 h for efficient loading.

Lyophilization technique: The above mentioned limitations can be overcome by employing a simple and cost-effective method in which water/tert-butanol mixture is used for dissolving drug as well as polymer and then the solution is lyophilized. Drug-loaded polymeric micelles are then obtained by redispersing the lyophilized product in a suitable vehicle [30, 31].

16.6.1 Drug Loading Capacity of Polymer Micelles

Almost one-third of newly discovered drugs are highly insoluble in water, but there is no standard method to solubilize such drugs [32]. As a result of the capability to load lipophilic molecules into the hydrophobic core, polymer micelles have been widely used to solubilize and deliver poorly water-soluble drugs (Fig. 16.2).

16.6.2 Theories of Drug Solubilization by Polymeric Micelles

The first-generation polymer micelle were used to solubilize (or load) highly lipophilic drugs like paclitaxel using PEG-b-poly(D,L-lactic acid) (PDLLA or PLA) as polymer. As reported the loading capacity of paclitaxel is ~ 10–20 % (wt/wt) [17].

This enhancement of drug solubility in water, which is derived from hydrophobic interaction between hydrophobic polymer blocks and drugs [33]. The hydrophobic interaction, more exactly hydrophobic effect, is a phenomenon induced by the London dispersive force that exists between any kinds of molecule. The hydrophobic effect is induced when hydrophobic molecules are mixed with water, because the London dispersive force between lipophilic drugs and hydrophobic blocks is much stronger than that between the lipophilic drug and water.

Although the hydrophobic effect is a major driving force, drug loading capacity and efficiency also depend on the miscibility between polymers and drugs. The mechanism of drug loading into a polymer micelle is explained by the Hildebrand–Scatchard solubility parameter (δ),

$$\delta = \left(\Delta E_{\text{vap}} / V\right)^{0.5}$$

where ΔE_{vap} is the energy of vaporization and V is the molar volume of the solvent [34].

The drug loading into a polymer micelle is in a way mixing of the polymer with drugs, the loading capacity can be described by the Flory–Huggins theory, expressed by

$$\chi_{\text{drug}-\text{polymer}} = \left(V_{\text{drug}} / RT\right)\left(\delta_{\text{drug}} - \delta_{\text{polymer}}\right)^2$$

where $\chi_{\text{drug-polymer}}$ is the Flory–Huggins interaction parameter between the drug and the polymer, V drug is the volume of the drug, R is the ideal gas constant, T is the temperature, and δ_{drug} and δ_{polymer} are the Hildebrand–Scatchard solubility parameters of the drug and the polymer, respectively [35, 36].

The above equation describes the miscibility between polymers and drugs. Letchford and colleagues investigated the miscibility of PEG-b-PCL with five different drugs [37] and observed that etoposide, paclitaxel, plumbagin, curcumin, and indomethacin followed the ascending order of $\chi_{\text{drug-polymer}}$. As a lower value (<0.5) of the Flory–Huggins parameter means a better solubility, indomethacin is the drug best solubilized in PEG-b-PCL micelle among the five. It should be noted that the hydrophobicity of each drug does not follow the order of the Flory–Huggins parameter, indicating that a hydrophobic effect is not the only mechanism to explain the efficiency of drug loading into polymer micelles. Similarly, the heat of mixing is another parameter to describe the miscibility between polymers and drugs. The drug loading efficiency was found to be highly dependent on the heat of mixing and the order was poly(benzyl-L-aspartate) (PBLA) >PCL > PDLLA> PGA. It is therefore obvious that the drug loading in to a polymer micelle is not only forced by the hydrophobic effect, but also facilitated by other interactions between polymers and drugs to increase the miscibility.

In addition, another important parameter to govern the drug loading capacity of polymer micelles is the hydrophilic–lipophilic balance (HLB) of block copolymers. For example, block copolymers with longer hydrophobic block showed better drug

loading property, which was confirmed by determining the partition coefficient of drugs into PEG-b-PCL micelles [37].

The hydrotropy also significantly enhances the aqueous solubility of poorly soluble compounds [38, 39]. The mechanism of hydrotropic solubilization has been explained in several ways, such as hydrophobic effect, hydrogen bonding and stacking interaction [38]. The hydrotropes form non-covalent aggregates only above a certain concentration, which is called the minimal hydrotrope concentration (MHC) [40]. As hydrotropes are small molecules containing both hydrophobic and hydrophilic moieties, the hydrophobic effect can be a driving force to generate aggregates such as surfactants. In addition, polar groups of the hydrophilic moieties can interact with drugs by means of hydrogen bonding [41]. Hypothetically, hydrotropes may break the hydrogen bonding between drugs that is considered as one of the drug crystallization mechanisms.

Most hydrotropes have an aromatic ring substituted by heteroatoms. Depending on the substituted atom species, the benzene rings can interact with each other and be stacked (Π– Π stacking) [42, 43]. As most poorly soluble drugs consist of one or multiple benzene rings and polar groups, the self-aggregation property of hydrotropes can be expanded to complexation between hydrotropes and lipophilic drugs [44, 45].

The significance of multiple interaction parameters in describing the solubilization phenomenon is indicated in the linear solvent free energy relationship (LSER) equation [43],

$$\log \mathrm{SP} = c + rR_2 + s \quad {}_2 + a\Sigma a_2 + b\Sigma\beta_2 + v\mathrm{Vx}$$

where SP is the property of interest for a drug (i.e., partition coefficient), R_2 is the excess molar refraction of the solution derived from the London dispersion force, p_2 is the drug dipolarity/polarizability, Σa_2 is the hydrogen bonding acidity of the drug, $\Sigma\beta_2$is the hydrogen bonding basicity of the drug, and Vx is the McGowan's characteristic volume calculated from molecular structure. The c, r, s, a, b, and v are regression coefficients.

Based on the LSER theory, drug partition in two immiscible phases of water and micelle core-forming polymer is explained by transferring the free energy of drugs in water to that in polymer. This free energy is proportional to the sum of multiple independent interactions. Therefore, the LSER equation suggests that the important parameters to maximize the miscibility are hydrophobicity, electrostatic interaction, dipole–dipole interaction, hydrogen bonding, and size of the drug. When the miscibility between polymers and drugs is optimized, it is expected that much improved drug loading capacity of polymer micelles will be obtained. Introduction of the hydrotropy into polymer micelles is one solution to accomplish the optimized miscibility.

16.6.3 Stability Issues

It is known that polymeric micelles possess high structural stability provided by the entanglement of polymer chains in the inner core. This stability has two aspects: static and dynamic [46–49]. Static stability is described by a critical micelle

concentration (CMC). Generally, polymeric micelles show very low CMC values in a range from 1 mg/mL to 10 mg/mL. These values are much smaller than typical CMC values of micelles forming from low-molecular weight surfactants. The second aspect, dynamic stability, is described by the low dissociation rates of micelles

Intravenous injections of polymeric micelles could get destabilized due to extreme dilutions they undergo in blood. This would lead to leakage and burst release of loaded drugs. Such problems could be overcome by improving the interaction between the drug and polymer in the shell via chemical conjugation or by cross-linking [50]. Other causes for decrease in stability of polymeric micelle are: Dis-balance in the HLB of the system due to over-loading of hydrophobic moiety (drug) into the core region; Hydrolytic cleavage of drugs or copolymers in aqueous systems. However, lyophilized polymeric micelle formulations have shown to possess improved long-term stability for intravenously administered preparations [51]. Other strategy used for stabilization is cross-linking of hydrophilic shell and/or hydrophobic core.

16.6.3.1 Cross-Linking the Shell

Cross-linking of hydrophilic shell leads to a stabilization of the micelle system and delays the degradation of the micelle. This strategy involves introduction of cross-linkable groups within the hydrophilic portion of the copolymer and then using polymer chemistry to cross-link the hydrophilic shell portion after the micellization of the polymer. Other approaches, such as conjugation of the core with the drug, can also be useful in preparing sustained-release micellar systems. For example, multifunctional, multi-armed PEG can be copolymerized with some degradable hydrophobic polymers to form an amphiphilic block. PEG branching in this polymer can be used to create cross-linkable groups in the system to prepare a shell cross-linked micelle system. Thurmond et al. [52] prepared micelles from polyvinyl pyridine-b-poly styrene (M_n = 52,500 Da) block copolymer, which on self-assembly, forms shell cross-linked knedel-like micelles. Li et al. reported similar stabilization of the micelles by cross-linking the shell of the micelles made from a poly(styrene-b-butadiene-b-styrene) polymer [53] by first preparing micelles in aqueous medium and then cross-linking the hydrophobic portion of the micelles using chloromethylation and amination.

16.6.3.2 Cross-Linking the Core

Shell cross-linking strategies needs preparation at a high dilution to avoid inter micelle cross-link formation which decreases the efficiency of the process [54]. Hence, the strategy of core cross-linking was developed to form a core matrix that traps the drug inside it, thereby controlling the diffusion of the drug from the core. Many approaches have been tried to stabilize the core by cross-linking it with different functional groups. Thiol group can be introduced to cross-link the core with

a disulfide group and the cross-linked core polymer be used to prepare polyion complex micelles. In such a polyion complex, the electrostatic interaction between two polymer segments drives association. Kazikawa et al. synthesized micelles using PEG-5,000-b-poly(lysine) diblock copolymer [55]. The cross-linking of the poly(lysine) core was achieved using thiolation chemistry. A completely biodegradable system was prepared by Hu et al. using the polymer PEG-b-PLA with a 5-methyl-5-allyloxycarbonyl-1,3-dioxane-2–1 (M_n = 4,500 Da) group as the polymerizable group for cross-linking the core [56]. The cross-linking was achieved post micellization by reaction with 2,2-azoisobutyronitrile. These micelles (130 nm size) were shown to survive water dilution and temperature better than non-cross-linked micelles. Core cross-linked strategy can also be used to prepare drug-loaded micelles that offer a longer sustained release than non-modified regular micelles.

16.6.3.3 Use of a Low Critical Solution Temperature Hydrogel

Low critical solution temperature (LCST) hydrogel can be used to stabilize the micelles by polymerizing LCST gel along with the core of the micelle to stabilize the core. LCST gels remain in a swollen state at room temperature, allowing drug loading while at physiological temperatures, these gels collapse and lock the hydrophobic portion of the micelle forming a locked core that contains the drug. Such a locked interpenetrating network in the core prevents the breakdown of the core upon dilution and also the drug loaded in the core would remain in the micelles for prolonged release. Such a system with pluronic micelles and an LCST gel was reported by Rapoport [57] where he suggested three ways to stabilize pluronic micelles, namely, core cross-linking, introducing vegetable oil in the hydrophobic portion to stabilize the micelles and polymerizing an LCST gel with the hydrophobic portion of the micelle to stabilize the core. However, one major disadvantage of using an LCST gel in the core of the micelle is that it increases the micellar size by severalfold. Rapoport reported a size increase from 12–15 nm to 30–400 nm [57].

16.7 Characterization of Polymeric Micelles

16.7.1 Critical Micelle Concentration (CMC)

Amphiphilic polymers exist in the form of micelles in aqueous media and when the delivery system gets diluted below CMC the micelles may collapse. Hence, CMC is the critical parameter for the formation and the static stability of polymeric micelles. Some of the methods used for determination of CMC in aqueous dispersions of micelles include surface tension measurements, chromatography, light scattering, small angle neutron scattering, small angle X-ray scattering, differential scanning calorimetry, viscometry, and utilization of fluorescent probes. However, the simplest method is by plotting the surface tension as a function of the logarithm of the concentration.

The CMC is said to be attained when the surface tension stops decreasing and reaches a plateau value. Use of pyrene as a fluorescent probe for estimating CMC is also much reported [58].

16.7.2 Size and Shape Determination

Polydispersity index of the prepared micellar solutions are best studies by quasi-elastic light scattering technique. Monodisperse micelles produce blue color from light scattering while the scattered light is white for aggregates [59]. Size of polymeric micelles usually falls in the colloidal range and so scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques have been widely used for the direct visualization, size and shape determination of particularly block copolymer micelles. Recently, cryo-TEM technique has increasingly started gaining importance for characterization of block copolymer micelles in aqueous medium. SEM or atomic force microscopy (AFM) reveals information regarding size distribution when chemically attached micelles to surfaces are presented. Hydrodynamic diameters and poly-dispersity indices of micelles are also obtained using photon correlation spectroscopy. Recently size characterization of drug-loaded polymeric micelles using asymmetrical flow field-flow fractionation and the structure of assemblies by small angle neutron scattering are also reported [60, 61].

16.7.3 In Vitro Drug Release Behavior

In vitro drug release behavior from micelles is studied by placing the micellar solution in a dialysis tube immersed into a flask containing release medium, kept at a constant temperature. At predetermined time intervals, aliquots of the release medium are taken and replaced by fresh medium. The content of drug released in the medium are measured by spectroscopic or other suitable method [62]. The parameters affecting the drug release are;

16.7.3.1 Micelle Stability

Dissociation of micelles into single chains will obviously free the entrapped molecules. Similarly, erosion or biodegradation of the carrier could provoke the escape of drug molecules. However, biodegradation of polymeric micelle follow controlled degradation and hence may release the entrapped drug molecules in a sustained manner [63].

In cases of stable polymeric micelle that are slowly biodegradable or non-biodegradable and above the CMC, the drug release will depend on the rate of diffusion from the micelle. The rate of diffusion was shown to be influenced by various factors mentioned below;

- The core-forming block length—increase in the length of the core forming block will favor slow and continuous release as the drugs located in the core have to diffuse through a longer path [64].
- The micelle morphology—spherical, cylindrical, bilayer, etc. are associated with different diameters and surface (and interface) areas per micelle, thus affecting the release rate.
- The physical state of the core—physical state of the micelle core, whether solid-like or liquid-like, was also shown to influence micelle stability and, in parallel, the release of the entrapped drug. For example, diffusion of a drug through a glassy core was slower than through a more mobile core [65] and this tendency could be qualitatively correlated with the respective glass transition temperatures of the core components.
- The presence of cross-links (within either the core or the corona segments), and the compatibility of the copolymer–drug pair cross-linking of the corona-forming blocks affects the permeability of the corona and the period within which drugs diffuse [66].
- The polymer/drug compatibility–polymer/drug compatibility can also influence drug release and drug incorporation. The Flory–Huggins interaction parameter can be used to infer the characteristics of drug release of a system. Generally speaking, the stronger the interaction between the drug and the core-forming block, the slower its release from the micelle.
- Drug localization—the localization of the drug within the micellar assembly is expected to influence the release, with molecules located at the core/corona interface or within the corona diffusing faster than those located in the core [65].

16.7.3.2 Drug Properties

Properties inherent to the drug molecule such as its molecular volume, physical state in the micelle core, relationship between the molecular volume of the drug and diffusion constant are other parameters to influence the drug release. The physical state of a drug in the micelle core can alter the drug release profile. Jeong et al. [64] showed that an increase in the amount of clonazepam loaded into PEG-b-PBLG micelles (12.1–32.8 % (*w/w*)) resulted in as lower drug release. Differential scanning calorimetry thermograms revealed that crystallization of the drug occurred at the higher loading.

Drug release profiles determined in vitro are useful to compare drug formulations but they rarely correlate with the in vivo behavior. Most of the time, the release gets accelerated in vivo. For example, the in vitro release of hydroxylcamptothecin

loaded in PEG-b-PCL micelles occurred over several days while the drug was cleared from plasma within few hours following i.v. administration of the micelles [67]. Similar results of premature micelle disassembly were reported [68, 69] where the loss of integrity of polymeric micelle within an hour after intramuscular or subcutaneous injection was observed. It was recently demonstrated that the destabilization of the polymeric micelle and the release of the loaded drug in vivo is possibly due to interactions with plasma proteins [70]. In addition, factors of in vivo micelle destabilization such as interaction with other blood components, the translocation of hydrophobic drugs to the lipid components in the blood and the degradation of the copolymers, can also contribute to the disassembly of the micelles and the fast release of the drug.

16.7.3.3 Drug Release from Specialized Micellar Systems

In the case of polyion complex micelles (PICM), drug release is not diffusion-based but rather occurs through dissociation of electrostatic interactive forces between oppositely charged ions, of the assemblies. The dissociation in vivo, mostly takes place by exchange events with charged ions (i.e., salts, heparin). Similarly, the dissociation of the polymer–metal complex micelles occurs through the substitution of the metal from the coordinating groups of the copolymer by ions in the medium, thus resulting in the micellar dissociation and subsequent release of the drug.

In addition to the above mentioned mechanisms, the drug release from the micellar carriers are also triggered by a change in pH, temperature or in the redox state of the surrounding medium. Ultrasounds have also been utilized to trigger drug release from micellar systems in vitro and in vivo [71, 72].

pH-Triggered Drug Release

pH variations occur at different pathological/physiological sites permitting drug release with change in the environmental pH. For example, the microenvironment pH in tumors is generally more acidic than in normal tissues [73]. Changes in pH are also encountered upon cellular internalization of the drug-loaded carriers via clathrin-mediated endocytosis, resulting in an increase of acidity inside the endosome. Orally administered formulations experience a pH gradient as they transit from the stomach to the jejunum. Several strategies have been exploited to achieve pH-sensitivity, most of which are based on changes in the polymer properties following the protonation/deprotonation of acidic and basic groups present along the polymer chain or on the hydrolytic cleavage of hydrophobic functionalities or cross-links.

For example, polymeric micelle with a corona composed of a PNIPAM copolymer bearing carboxylic acid functionalities show the pH-sensitivity. At neutral pH, the carboxylic acid groups make the PNIPAM segment soluble but a sharp decrease in the solubility of the corona is observed as the pH is lowered. As a result, mixing

of the PNIPAM chains and core region takes place, increasing the polarity of the core and promoting the release of the entrapped drug [74]. Another approach is to impart amphiphilicity to a copolymer by the conjugation of hydrophobic moieties to one of the polymer block (the core block) via pH-sensitive links. Decrease in pH result in the hydrolysis of the link and exposes polar groups on the core forming block, resulting in the destabilization of the micelles and drug release. A system like this was reported by Gillies et al. [75, 76] who developed block copolymers of PEG and either PLL or polyester dendrons and used highly acid-sensitive cyclic acetals to attached hydrophobic groups to the dendrimer periphery. These polymers self-assembled into micelles that were stable in neutral aqueous solution but disintegrated into unimers at mildly acidic pH [65] following loss of the hydrophobic groups upon acetal hydrolysis [76]. The pH-sensitive micelles were produced by directly conjugating a hydrophobic anticancer agent (DOX) to copolymers to release the drug at acidic pH found in tumor tissues [75].

Temperature Sensitivity

Local hyperthermia is observed in some disease states and local increases in body temperature can also be induced by exterior means, making temperature-directed drug release as another viable strategy for localized drug release. To achieve this, polymers presenting a lower critical solution temperature (LCST) transition like PNIPAM can be incorporated in the composition of micelles. The LCST of PNIPAM can be adjusted within a desired range by copolymerizing it with hydrophilic or hydrophobic monomers which strengthen or weaken the interactions between polymer chains and water, resulting in an increase or decrease in water solubility, respectively [77–79]. Below the LCST (at normal temperature), the non-polar core is segregated from the hydrated PNIPAM corona while, at higher temperature (above the LCST), corona collapses and results in the increased mixing of the NIPAM corona units and hydrophobic core units. This increases the core polarity triggers release of the drug incorporated in the micelle [74, 77, 80, 81].

Redox Sensitivity

Another stimulus to trigger the release of drugs from the polymeric carriers is redox sensitivity due to the presence of oxygen-reactive species released by activated macrophages in the inflamed tissues and certain tumors [82]. The hydrophobic poly(propylene sulfide) (PPS) in PEG-b-PPS block copolymer respond to such oxidative condition and readily gets converted to hydrophilic poly(sulfoxide) or poly(sulfone) by mild oxidizing agents. Micelles obtained from this polymer was demonstrated to release the incorporated hydrophobic drugs during the solubilization or swelling of the polysulfide upon oxidation [83, 84]. Another mechanism could be to take advantage of the reductive conditions met in the cytosol which could cleave disulphide bond generally used to link drugs or siRNA to polymer like PEG. This cleavage eventually causes release of the drug in cytosol [85, 86].

16.8 Micelle–Cell Interaction and In Vivo Fate of Polymeric Micelle

A polymer micelle hardly interacts with cell membrane if its hydrophilic corona is biologically inert but the hydrophilic shell of polymer micelles is not totally inert. It is well known that polymer micelles enter cells by means of endocytosis by specific or nonspecific interaction [87]. The mechanism explaining endocytosis of polymer micelles has not been fully clarified. One possibility based on the role of the labeled dye is charge on the micelle. It is known that positively charged macromolecules can be effectively internalized by electrostatic interaction with heparan sulfate on cell surface [88].On the other hand, polymer micelles are shown to increase the drug accumulation inside cells without endocytosis also. For example, polymer micelle made of PluronicP-85 or P-105 enabled effective accumulation of a hydrophobic dye inside cells by inhibiting *P*-glycoprotein (*P*-gp) [89, 90]. A polymer micelle consisting of PEG-b-PCL also showed a similar effect on blocking the *P*-gp function [91].

Expression of targeting moieties onto micelle surface provides a major strategy to enhance the therapeutic effect of micellar drug carriers. Micelles conjugated with different targeting moieties such as biotin, folate, antibodies, growth factors, or homing peptide shave been developed especially for intracellular delivery of anticancer drugs [92]. However, most of the micelles are based on the physical assembly of block copolymers so their stability in blood is not guaranteed. Therefore, improving the micelle stability in blood should be considered in order to optimize the active targeting strategy using targeting moieties.

In vivo pharmacokinetics and pharmacodynamics of drugs formulated using polymer micelles have been widely studied, using radioisotope as a tool to monitor the biodistribution. Polymeric micelles are mostly located in liver, kidney, spleen, and blood indicating their capability for prolonging circulation time in blood, which is an important rationale to develop micellar formulation of lipophilic drugs. Table 16.3 demonstrates that micelles (or unimers) are highly distributed to organs that have excretion and metabolism functions.

16.9 Applications of Polymeric Micelle

Most drug carrier applications have been studied with AB- or ABA-type block copolymers because the close relationship between micelles' properties and the structure of polymers can be evaluated more easily with AB- or ABA-type block copolymers than with the other types of copolymers.

Advantages of polymeric micelle as a drug carrier

Advantages of polymeric micelles as drug carriers are:

1. Very small size (diameter ¼ 10e100 nm)
2. High structural stability

Table 16.3 Studies on biodistribution of polymer micelles after intravenous administration

Polymer	Block ratio	Micelle Size (nm)	Model	Biodistribution	Reference
PEG-b-PCL	6,000–1,000	60	Mice	Blood > bone > kidney > liver > lung > brain	[93]
	5,000–5,000	56	Mice	Liver > kidney > spleen, lung > heart	[94]
	21,000–10,000	190	Rats	Liver, spleen > lung, heart	[95]
	5,000–13,000	99–118	Rats	Blood > heart > plasma > liver > kidney > spleen	[96]
	2,000–2,400	25	Mice	Too bright background fluorescence from mice	[68]
PEG-b-PLA	5,000–7,000	300	Rats	Blood > bowel (inflamed site) > liver > kidney > spleen	[97]
	2,000–1,750	220	B-16 T.B* mice	Tumor > liver > kidney, spleen > lung, heart > plasma	[98]
	5,000–5,000	300	Rats	Blood > liver > kidney > lung, heart, spleen	[97]
	5,100–5,300	37–38	Mice	Liver > kidney > lung, spleen	[99]
	33,000	128	MDA-MB-435-TB* mice	Kidney > liver > lung > spleen > heart > tumor > muscle > blood	[100]
PEG-b-PAsp-DOX	4,300–1,900	50	Mice	Blood > spleen > kidney > liver > lung > heart	[59]
PEG-b-P(Asp(Bz-70)	5,000–4,700	192	C26 TB* Mice	Spleen, tumor, plasma > kidney, lung > heart	[101]
PEG-b-P(Asp(Bz-75)		19	Mice	Liver > kidney > lung > spleen	[102]

(continued)

Table 16.3 (continued)

Polymer	Block ratio	Micelle Size (nm)	Model	Biodistribution	Reference
PEO-b-PPO-b-PEO	1,150–2,300–1,150 (P85)	15	Mice	Liver > spleen > kidney > lung > brain	[103]
	1,125–3,250–1,125 (P105)	24	Rats	Lung > spleen > kidney > liver > ovary > heart > blood	[104]
PVA-b-PLA	2,480–1,820	16–17	C26 TB* Mice	Liver > kidney, lung > spleen > heart > muscle	[105]
Micelles with targeting ligands RGD-PEG-b-PLA	33,000	128	MDA-MB-435-TB* mice	Kidney > liver > tumor > lung, spleen > heart > muscle > blood	[100]
FA-PEG-b-P(Asp-Hyd-DOX)		62–96	KB-bearing mice	Lower FA content: blood > liver, spleen > tumor > kidney, heart; Higher FA content : liver > blood, spleen > tumor > kidney, heart	[106]
Crosslinked micelles PAA-b-PMAA	4,800–18,600	24	Mice	Spleen > liver > kidney > blood > lung	[107]
PEG-b-P(HEMAm-Lac)		53	14C-bearing mice	Non-crosslinked micelle: liver > tumor > skin > spleen > blood Crosslinked micelle: liver > blood > skin, tumor > spleen	[108]

*TB** Tumor bearing, *ATRA* All-trans retinoic acid, *Bz* Benzyl, *CsA* Cyclosporine A, *CPT* Camptothecin, *DOX* Doxorubicin, *F-5-CADA* Fluorescein-5-carbonyl azide diacetate, *FA* Folate, *Glu* Glutamic acid, *HEMAm N*-(2-hydroxyethyl) methacrylamide, *Hyd* Hydrazone, *Lac* Lactate, *N/A* Not applied, *PAsp* Poly(aspartic acid), *PCL* Poly(e-caprolactone), *PEG* Poly(ethylene glycol), *PEO* Poly(ethylene oxide), *PET* Positron emission tomography, *PLA* Poly(DL-lactide), *PMMA* Poly(methacrylate), *PPO* Poly(propylene oxide), *PTX* Paclitaxel, *PVA* Poly(vinyl alcohol), *Tyr* Tyrosine

3. Large amount of drug loading
4. High water solubility
5. Low toxicity
6. Incorporation of various chemical species

(a) Polymeric micelles are formed typically in a diameter range from 10 nm to 100 nm with a substantial narrow distribution. This size range is considered ideal for the attainment of stable, long-term circulation of the carrier system in the bloodstream. The small size of polymeric micelles is also a big benefit in the sterilization processes in pharmaceutical productions.

(b) Polymeric micelles possess high structural stability provided by the entanglement of polymer chains in the inner core. This stability has two aspects: static and dynamic [46–49]. Static stability is by a critical micelle concentration (CMC). Generally, polymeric micelles show very low CMC values in a range from 1 mg/mL to 10 mg/mL. These values are much smaller than typical CMC values of micelles forming from low-molecular weight surfactants. The second aspect, dynamic stability, is described by the low dissociation rates of micelles, and this aspect may be more important than the static one for in vivo drug delivery in physiological environments that are in non-equilibrium conditions.

(c) Polymeric micelle carrier system as a drug carrier has advantage of high water solubility even when hydrophobic drugs are incorporated [109]. Generally, in conventional synthetic polymer–drug conjugate systems and antibody–drug conjugate systems, a loss of the carrier's water solubility resulting from the conjugation of a hydrophobic drug creates a serious problem. Several research groups reported this problem of the polymer–drug conjugates in syntheses [110–112] and in their intravenous injections [113].

(d) Polymeric micelles can incorporate a large number of hydrophobic drug molecules in the micelles' inner core, and simultaneously, the micelles can maintain their water solubility by inhibiting intermicellar aggregation of the hydrophobic cores with a hydrophilic outer shell layer that works as a barrier against intermicellar aggregation. This is a great advantage because many potent drugs that have been developed in recent years are very hydrophobic and are, therefore, water insoluble.

(e) Polymeric surfactants are known to be less toxic than low-molecular-weight surfactants, such as sodium dodecyl sulfate. Furthermore, in theory, polymeric micelles are considered very safe in relation to chronic toxicity. Possessing a much larger size than that for critical filtration in the kidney, polymeric micelles can evade renal filtration, even if the molecular weight of the constituting block copolymer is lower than the critical molecular weight for renal filtration. On the other hand, all polymer chains can be dissociated (as single polymer chains) from the micelles over a long time period. This phenomenon results in the complete excretion of the block copolymers from the renal route if the polymer chains are designed with a lower molecular weight than the critical value for renal filtration.

(f) The sixth advantage is the fact that various chemical species can be incorporated into polymeric micelles. As explained previously, the most commonly examined chemical species are hydrophobic low-molecular-weight organic compound drugs. These drugs can be incorporated into the micelle inner core either by chemical conjugation to the inner-core-forming polymer block or by physical entrapment owing to hydrophobic interactions between the entrapped drug molecules and the hydrophobic inner-core forming polymer block. Hydrophobic interactions also work as a driving force for micelle formation. On the other hand, polymeric micelles are formed through ionic interactions between charged polymer chains. For example, polymeric micelles form from poly(ethylene glycol) (PEG)-b-poly(lysine) block copolymers and poly(aspartic acid) (ASP) homopolymers where the poly(lysine) chain is positively charged and the poly(ASP) chain is negatively charged. If negatively charged polypeptides [114] or nucleic acid [115] are used in place of poly(ASP), these pharmacologically active macromolecules are incorporated into polymeric micelles for protein, gene, and small interfering RNA delivery purposes. Furthermore, metal ions or metal ions' chelates can be incorporated into polymeric micelles through coordination bonds or ionic interactions. A platinum chelate cisplatin, which is a widely used anticancer drug, was successfully incorporated into polymeric micelles forming from PEG-b-poly(ASP) through a ligand exchange reaction between a carboxylic acid residue of the poly(ASP) chain and a chloride ion of cisplatin [116, 117]. Alternatively, gadolinium (Gd) ions, which can work as a magnetic resonance imaging (MRI) contrast agent, were incorporated into polymeric micelles by the use of a chelatemoiety-conjugated block copolymer [118, 119]. As stated above, various pharmaceutical drugs, genes, and contrast agents can be incorporated into polymeric micelles with appropriate choices of block copolymer structures.

Disadvantages of polymeric micelle as a drug carrier
Disadvantages of polymeric micelle drug carriers are:

1. Specific disadvantages of polymeric micelle carriers
   (a) Difficult polymer synthesis
   (b) Immature drug-incorporation technology
2. Common disadvantages of polymeric carriers
   (a) Slow extravasation
   (b) Possible chronic liver toxicity due to slow metabolic process

The first disadvantage is a fact that relatively high levels of polymer chemistry are needed in the polymeric micelle studies. AB type of block copolymer is one of the most favorable structures for polymeric micelle carriers. The architecture of the AB block copolymer is very simple. However, its synthesis is more difficult than that of random polymers particularly in large industrial scale.

The second disadvantage of the polymeric micelle systems is the immature technology for drug incorporation in a physical manner. Yokoyama et al. reported that physical incorporation efficiencies is dependent on various factors in drug-incorporation processes [116]. Presently, there seem to be no universal incorporation method applicable to any polymer. Furthermore, in some methods the drug incorporation may be difficult on a large industrial scale.

The third disadvantage is much slower extravasation of polymeric carrier systems than that of low-molecular weight drugs. The polymeric systems translocate from the bloodstream to the interstitial space of organs and tissues through intracellular channels and inter-cellular junctions, whereas the drugs permeate directly through lipid bilayer cell membranes. Therefore, a long circulation character of the polymeric systems is an essential requirement for delivery of a therapeutic amount owing to compensation of the slow extravasation with a large area under the curve (AUC) value that results from the long circulation.

The forth disadvantage is a risk of chronic liver toxicity. Drugs conjugated or incorporated in the polymeric carrier systems are metabolized in liver in a slower manner than free drug, since access of metabolic enzymes to drugs is inhibited because of the conjugation and incorporation. Therefore, toxic side effects of the conjugated and incorporated drug may be exhibited for a longer period than a case of free drug whose toxic effects can be lowered through metabolism in a short period.

16.9.1 Drug Solubilization

The micellar core is a compatible microenvironment for incorporating water-insoluble guest molecules as the hydrophobic molecules can be covalently coupled to the block copolymers or physically incorporated into the hydrophobic core of micelles. The solubilization process leads to enhancement of their water solubility and thereby bioavailability [57]. The extent of solubilization depends upon the process of micelle preparation, the compatibility between the drug and the core forming block, chain length of the hydrophobic block, concentration of polymer, and temperature [120]. Above CMC of the polymer, there is a sharp increase in the solubility of drug as it gets more space to occupy in the aggregates of the hydrophobic part of the micelle. However, the core region has limited capacity for accommodation, for instance, Pluronic P85 has a core region which is 13 % of the whole micelle weight [121]. The influence of hydrophobic block length on solubilization of griseofulvin in polyoxy ethylene and polyoxy butylene copolymer micelles investigated showed that the solubilization capacity was dependent on the hydrophobic block length up to a certain extent (15 units of hydrophobic block), after which the solubilization capacity became independent of the same [122]. Dong and coworkers also concluded that solubilization capacity of polyurethane surfactants increased with an increase in the hydrophobic segment of the diblock and triblock polyurethane surfactants [123].

Table 16.4 Different approaches used for extended release through polymeric micelles

Approach	Polymer used	Drug	Duration of in-vitro release	Comments
Prodrug synthesis	PEG-PCL	Paclitaxel	Up to 14 days	Release varies with the prodrug chemistry
Drug–polymer conjugate	PEG-PLGA	Doxorubicin	15 days	Loading efficiency was almost 100 % with drug

16.9.2 Sustained Release Using Polymeric Micelle

Current approaches to achieve sustained drug release from the micelles include use of prodrug synthesis, novel polymers, layer by layer assembly of micelles on a solid support, reverse micelles, drug–polymer conjugate micelles, and polymer films that form micelles in vivo. However, the sustained release achieved by these strategies lasts only up to a maximum of few weeks.

16.9.2.1 Prodrug/Drug Polymer Conjugate Micelle

In this approach a prodrug that is most compatible with the micelle-forming amphiphilic molecule is desirable. The two limiting processes controlling drug release are prodrug release from the micelles and prodrug conversion to drug. One such example is paclitaxel palmitate, a paclitaxel prodrug, which was encapsulated in PEG-b-polycaprolactone (PEG-b-PCL) (M_w of PEG: 5,000, M_w of PCL: 10,500) micelles [124]. Other examples of this approach are summarized in Table 16.4.

16.9.2.2 Novel Polymers

This is the most common approach used to prepare sustained release micelles. Polymers with very low CMC (<0.1 μg/ml) can be used for prolonging the circulation time before the micelle degrades. The micelles undergo dilution in the body after intravenous injection leading to drop in the concentration of the polymer or surfactant below the CMC if the CMC value is higher. Therefore, a higher concentration of the polymer or surfactant has to be used to prepare the micelles so that they can withstand the dilution in the blood. But, in most cases, the use of high concentrations is not feasible due to toxicity related dose limitations. If the polymer or surfactant has a CMC lower than 0.1 μg/ml, concentrations as low as 5 mg/ml may be used to prepare a micelle formulation in order to counter the dilution effects in the blood. A variety of polymers including diblock copolymers, triblock copolymers and graft copolymers have been investigated for this purpose. Some polymers investigated for sustained release micelle are listed below.

Block Copolymers with Lipids

Block copolymers between a polymer and a lipid are one useful approach in preparing micelles. Lipids are more hydrophobic than most polymers and hence, a micelle made with a lipid as its hydrophobic part might lower the CMC. Hence, using fatty acyl chains as hydrophobic segments in an amphiphilic copolymer might be a useful approach. Further, it was observed that increasing the length of the hydrophobic portion of a micelle will lead to a decrease in its CMC [125]. Among diblock copolymer, distearoyl phosphatidyl ethanolamine (DSPE) has been used as the hydrophobic block with hydrophilic polyethylene oxide (PEO) to form 22 nm micelles. These micelles sustained release of lipophilic beclomethasone dipropionate for up to 6 days [126]. Lavasanifar et al. prepared micelles of polyethylene oxide-poly[*N*-(6-hexyl stearate-*l*-aspartamide)] (PEO-PHSA) to encapsulate amphotericin B [127]. The release of encapsulated drug was sustained (20 % released in 1 h) while the plain drug was released within 10 min. The release depended inversely on the degree of fatty acid substitution in the core.

Block Copolymers with Cyclodextrins

Another approach for drug delivery is supramolecular polymeric micelles which involves non-covalent interactions between a macromolecular polymer, and a small polymer molecule (guest molecule). One such attempt was made using α-cyclodextrins (α-CDs) as the hydrophilic macromolecular host and PCL (Mn = 37,000) as the hydrophobic guest molecule [128]. The formed supramolecular polymeric micelles having a mean diameter of 30 nm resulted in sustained release of an anti-inflammatory drug up to 700 h.

Diblock Copolymer Micelles

Diblock polymer that can physically interact with the drug can result in drug retention and sustained release from such polymer micelles. If the drug can form hydrogen bonds with the core of the micelle, then the release obtained from the micelle will be much more sustained. Micelles prepared from PEG-b-poly-*l*-lactic acid (PEG-b-PLLA; Mw: 8,500 Da) and PEG-b-PCL (Mw: 10,050 Da) block copolymers showed sustained release of the loaded drug, quercetin for approximately 160 h [129].

Triblock Copolymer Micelles

A triblock copolymer with small hydrophobic ends and a long hydrophilic midsection can assemble to form flower-like micellar structure in aqueous environment. These flower-like micelles can dissolve the drug in the hydrophobic core and sustain drug release for long periods of time. Zero-order release of sulindac and tetracaine has been reported using PLA–PEO–PLA, triblock flower-like micelles (7–13 nm) for 20 days and 10 days respectively [80].

16.9.3 Drug Targeting Potentials of Polymeric Micelle

Drug targeting is defined as selective drug delivery to specific physiological sites like organs, tissues, or cells, where the drug's pharmacological activities are required. Drug-targeting strategies are classified as active targeting and passive targeting [130, 131]. Active targeting aims at an increase in the delivery of drugs to the target by using biologically specific interactions, such as antigen-antibody binding or by utilizing locally applied signals, such as heating and sonication. On the other hand, passive targeting is defined as a method whereby the physical and chemical properties of carrier systems increase the target/non-target ratio of the administered drug.

In most of the active targeting processes particularly for tumors, passive transfer phenomena precede biologically specific interactions except in cases of intravascular targets, such as vascular endothelial targeting. Most tumor targets are located in extra-vascular space. To reach these targets through the bloodstream, translocation through the vascular endothelium is a necessary step, followed by diffusion in the interstitial space.

16.9.3.1 Passive Drug Targeting to Solid Tumors

The passive targeting of polymeric micelles on solid tumors can be achieved owing to the enhanced permeability and retention effect (EPR effect). Vascular permeability of tumor tissues is enhanced due to malformation of tumor vasculature during rapid angiogenesis and by the actions of secreted factors, such as kinin and vascular permeability factor. As a result of this increased vascular permeability, macromolecules selectively increase their transport from blood vessels to tumor tissues. Furthermore, the lymphatic drainage system does not operate effectively in tumor tissues. Therefore, macromolecules are selectively retained for a prolonged time in the tumor interstitium. However, the carrier systems must fulfill the following two requirements to avoid nonspecific capture at non-tumor sites:

1. The drug carrier systems must possess an appropriate size or molecular weight. The diameter of carriers must be smaller than approximately 200 nm if the reticuloendothelial system's uptake is to be evaded [132]. Additionally, molecular weights greater than a critical value (approximately 40,000) are favorable for evading renal filtration.
2. The drug carrier systems must not exhibit strong interactions or uptake with or by normal organs (especially the reticuloendothelial systems). These strong interactions and uptakes are typically seen for cationic [133] and hydrophobic carriers [134]. Therefore, the carrier systems should preferably possess hydrophilic surfaces, and their surface charge must be neutral or weakly negative. However, hydrophobic carriers can be used but hydrophilic coating using polymers like PEG is needed to avoid this interaction. Furthermore, the carrier systems must possess no other chemical structures that would be biologically recognizable to normal tissues.

Considering the two afore mentioned requirements, polymeric micelles are very much suited for passive targeting to tumors because they are formed in a diameter ranging from 10 nm to 100 nm. The second requirement can be easily fulfilled through a choice of hydrophilic and neutrally or weakly negatively charged polymers for the outer shell-forming block. With this choice, polymeric micelles can circulate in the bloodstream for a long time period by evading nonspecific capture, resulting in successful attainment of the EPR effect.

The first successful example of tumor targeting with a polymeric micelle carrier was reported by Yokoyama et al, where, doxorubicin (DOX) was chemically conjugated to ASP residues of PEG-poly(ASP) block copolymers (PEG-poly(Asp)) by amide bond formation and was presented as a polymeric micelle system [59, 135–137]. The PEG segment was hydrophilic, whereas the DOX conjugated poly(ASP) chain was hydrophobic. Therefore, the obtained drug-block copolymer conjugate (PEG-poly(Asp)-DOX) formed micellar structures owing to its amphiphilic character. In addition, DOX was also incorporated into the inner core by physical entrapment using hydrophobic interactions with the chemically conjugated DOX molecules. As a result, polymeric micelles containing both the chemically conjugated and the physically entrapped DOX in the inner core were obtained with the PEG outer shell. The DOX entrapped polymeric micelle circulated in the bloodstream for a long time and was delivered to the solid tumor site at much higher concentrations than that of free DOX [137].

Shiraishi et al prepared a polymeric micelle containing an MRI contrast agent using a poly(ethylene glycol)-b-poly(L-lysine) block copolymer derivative which was found to enhance MRI contrasts by shortening the T1 relaxation times of protons of water. This polymeric micelle was found to be targeted to a murine tumor C26, and the tumorwas successfully visualized with the targeted MRI contrast agent [119, 138].

16.9.3.2 Targeted Micelles

Selective biodistribution of micelles for improved efficiency could theoretically be achieved by using systems which respond to external stimuli like pH and temperature variations or by attaching specific ligands to the exposed hydrophilic ends of the carriers. These targeting mechanisms are referred to as active targeting.

pH-Responsive Micelles

Active targeting of drugs from polymeric micelle (PM) can be triggered by pH changes if the change in pH is associated to a pathological process like solid tumors presenting acidosis [139, 140]. Hence, micellar devices have been designed to trigger and/or enhance drug release in response to pH. An extended application of the pH-sensitive micelles has recently been introduced for the formulation of multiple anticancer agents in the context of combinatorial therapy for better patient

compliance and higher efficacy through a synergistic mechanism, thereby reducing the therapeutic dose and toxicity of the drugs. In this approach, pH-sensitive micelles are prepared using polymer conjugated with drugs at a precise ratio. For instance, Bae, Y. et al. [141] conjugated DOX and wortmannin to PEG-poly(aspartate hydrazide) through an acid-sensitive hydrazone bond. The polymer–drug conjugates then assembled into micelles in which the drug mixing ratio between DOX and wortmannin was critical. These mixed PM could reduce the DOX dose required for cytotoxicity through a synergistic drug action.

Temperature-Sensitive Micelles

The efficiency of the micellar carriers can also be improved by combining the EPR effect with temperature sensitivity. Thermo-responsive PMs were shown to release the loaded drug when the temperature increases beyond the lowest critical solution temperature (LCST), thereby increasing their therapeutic efficacy. For example, PM of (PNIPAM-*co*-*N*,*N* dimethyl-acrylamide)-b-poly(benzyl methacrylate) [80] and PNIPAM-b-poly(butyl methacrylate) (PNIPAM-b-PBMA) [142] loaded with DOX not only showed a thermo-responsive drug release behavior but also showed increased cytotoxicity towards bovine aortic endothelial cells in vitro above their LCST compared to the free drug.

Functionalized Micelles

The shell-components of PM, primarily selected to hinder nonspecific interactions and increase blood circulation times, may prevent internalization of the carriers by target cells [143]. Hence, systems presenting ligands at their water-exposed surface have been designed to enhance their selective binding to specific receptors on the cells and to promote the uptake of the drug loaded micelles by receptor-mediated endocytosis and enhance efficacy. For efficient targeting, the receptors must be overexpressed by target cells (e.g., tumor cells) compared to normal tissues [144]. A variety of molecules including antibodies [145, 146] peptides [147, 148], aptamers [149, 150], vitamins and sugar moieties [151] have been used to achieve targeting for anticancer drugs (Table 16.5) and genetic materials (Table 16.6).

The use of antibodies as the targeting moiety presents the advantage of selectivity, high affinity, and minimal competition for the receptor, contrary to what is observed with endogenous molecules such as folic acid (FOL) or transferrin [172, 173]. Antibodies, however, might induce immunogenicity, can be difficult to produce/handle and present a large size, potentially putting strain on micelle self-assembly. The use of small molecules such as sugars and vitamins can then become advantageous. As reported in many studies, it is important that the functional groups be readily available on the surface for efficient attachment to the receptors. The ligands can be attached either before or after the assembly of the particulate carrier.

Table 16.5 Examples of Polymeric micellar compositions loaded with various antitumor drugs

Polymer composition (targeting moiety)	Drug incorporated	Size (nm)	Reference
PEG-b-PLLA-DOX	DOX	90	[152]
Poloxamers 181 (PEG2-PPO30-PEG2) and 407 (PEG99-PPO65-PEG99)	DOX	21–27	[153, 154]
PEG-b-P(Asp)-DOX	DOX	40	[155]
Poloxamer 181 (PEG2-PPO30-PEG2)	DOX	–	[156]
PEG-b-PLGA-DOX and FOL-PEG-b-PLGA (Folic acid)	DOX	95–115	[157]
cRGD-PEG-b-PCL (cEGD)	DOX	20–27	[158]
PEG-b-P(Asp)-DOX	DOX	65	[159]
PNIPAM-b-PBMA	DOX	340	[77]
PEG-b-PDLLA	Paclitaxel	20–25	[160]
Galactose-PEG-b-PBLG (Galactose)	Paclitaxel	105	[161]
PEG-PE mAb 2C5 mAb 2G4	Paclitaxel	20	[162]
PEG-b-PBO or PEG-b-PSO	Docetaxel	16–21	[163]
P(NIPAM-*co*-MAA-*co*-ODA)	AlClPc	13–35	[81, 164]
P(NIPAM-*co*-VP-*co*-MAA-*co*-ODA)	AlClPc	20–34	[165]
PEG-b-P(Asp)	Cisplatin	20	[166–168]
PEG-PE	Dequalinium	–	[169]
PEG-b-poly(*N*-hexyl-L- spartamide)-stearic acid	Amphotericin B	–	[170]
PVP-b-PDLLA	Paclitaxel, Docetaxel, Indomethacin	40–100	[30, 90, 171]

Table 16.6 Examples of PM loaded with gene therapeutics (pDNA, AON, or siRNA)

Polymer composition (targeting moiety)	Genetic material incorporated	Size (nm)	Reference
PEG-SON/AON and PAMAM G5 and G3	AON	70	[178]
PEG-b-P(AEMA) and P(MAA-*co*-EA-*co*-BMA)	AON	30	[179]
AON-PLGA	AON (*c-myc*)	80	[63]
PEG-AON and PEI	AON (*c-raf*)	70	[180, 181]
PEG-AON and KALA	AON (*c-myb*)	70	[182]
FOL-PEG-AON and lipofectamine (Folic acid)	AON (GFP)	70–90	[183]
PEG-b-PLL	AON (*c*-Ha-*ras* or VEGF)	40–50	[184, 185]
PEG-siRNA and PEI	siRNA (VEGF)	–	[85]
PEG-siRNA and KALA	siRNA (VEGF)	<200	[86]
PEG-b-DPT	siRNA (luciferase)	–	[186]
Transferrin-PEG-cyclodextrin-containing polycations	Transferrin siRNA (*EWS-FLI1*)	50	[187]
cRGD-PEG-PEI (cRGD)	siRNA (VEGF)	100	[188]
Lactose-PEG-siRNA and PLL (Lactose)	siRNA (luciferase)	117	[189]
Fab′-PEG-PEI (Fab′)	pDNA (luciferase)	150	[146]

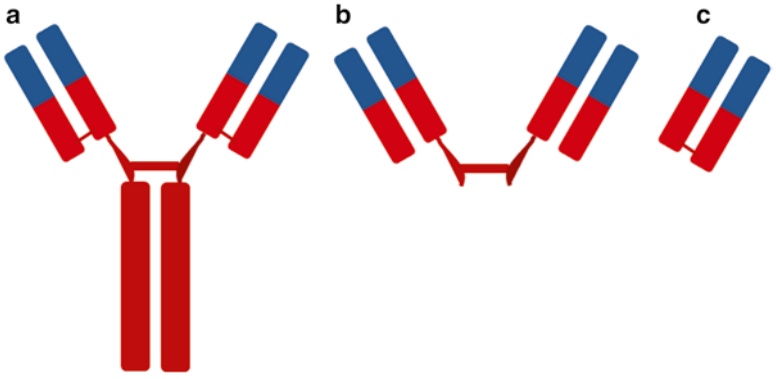

Fig. 16.3 Targeting can be achieved with the whole antibody (**a**) or its fragments such as F(ab′)2 (**b**), (Fab′) (**c**)

Monoclonal Antibodies and Antigen Binding Fragments

The whole antibody or its fragments such as the fragment antigen binding (Fab′) and F(ab′)2 or scFV (single-chain variable) can be used for targeting [174]. Using an antigen binding fragment (e.g., Fab′) instead of the whole monoclonal antibody provides an advantage of avoiding steric hindrance during complex formation due to its lower molecular weight (Fig. 16.3).

In addition, it can induce less immunogenicity when in vivo applications are sought. Micelles functionalized with either monoclonal antibody or Fab′ have both shown higher cellular uptake compared to non-targeted micelles. For instance, Merdan et al. [146] observed more than sixfold binding of the targeted micelles to the ovarian carcinoma cells (OVCAR-3 cells) using Fab′-PEG-PEI/pDNA PICM compared to the unmodified system. Increased cellular uptake and fourfold higher tumor accumulation of the drug (paclitaxel) in vivo was reported by Torchilin and coworkers [175] for whole antibody-PM targeting lung cancer cells.

Aptamers

Nucleic acid ligands (aptamers) have been used recently for the targeting of drug encapsulated PMs. Aptamers selected by screening a random library of nucleic acids to specific molecular targets can fold by intramolecular interactions into unique three-dimensional conformations capable of binding to target antigens with high affinity and specificity [176, 177]. An RNA aptamer targeting the prostate specific membrane antigen, overexpressed on prostate acinar epithelial cells was used to decorate PEG-b- PLA or PEG-b-PLGA micelles loaded with docetaxel [149, 150]. This targeted delivery system showed a marked increase in the cellular uptake and increased cytotoxicity in vitro and in increased antitumor efficacy in vivo over the non-targeted PM. However, the instability of DNA or RNA molecules in the blood may limit the use of these ligands in the clinic.

Non-immune Peptides and Proteins

Proteins like transferrin, an iron transporter, are used to target rapidly dividing cells such as tumor cells. Hu- Lieskovan et al. [187] developed a cyclodextrin-containing polycation that self-assembled with a siRNA inhibiting *EWS-FLI1* (a gene that is found in 85 % of patients with Ewing's tumor) for multicomponent delivery for metastatic tumor treatment. The surface of the complexes was then decorated with PEG and targeted with transferrin which downregulated onco-proteins and suppressed the spread of metastatic tumors up on systemic administration of the system. Transferrin, being a big molecule (80 kDa), can put strain on micelles and so a smaller peptide might be advantageous. One such example is the cyclic RGD (cRGD) peptide, a cellular transmembrane protein that has a marked role in tumor growth and metastasis and targets the αvβ3 integrin [190, 191]. The cRGD peptide was conjugated to maleimide-terminated PEG-b-PCL PM encapsulating DOX. The delivery system showed a threefold increase in cellular uptake when the surface density was adjusted to 5 % cRGD while, a more pronounced 30-fold increase was observed with 76 % cRGD attachment [158]. Similar studies with a PEGylated branched PEI modified with an RGD peptide at the distal end of the PEG further demonstrated the possibility of in vivo targeting with this peptide [188].

Vitamins

The expression levels of folic acid (FOL) receptors in tumors have been reported to be 100–300 times higher than those observed in normal tissues [144]. Functionalized PEG-b-PLGA chains with FOL covalently derivatized via its gamma-carboxyl group showed high affinity for the FOL receptor (especially for FOL receptors alpha) and retains its receptor binding affinity [157, 163, 192]. This polymeric system physically mixed with PEG-b-PLGA-DOX and free-DOX to produce targeted micelles decreased the tumor growth rate compared to control non-targeted micelles and enhanced the antitumor efficacy when administered at the same dose level.

Sugars

One of the most common cancers affecting human is the hepatocarcinoma and so the development of liver-targeted drug carriers is therefore highly desirable. Advances in this area rely on the fact that hepatocytes express carbohydrate receptors, i.e., asialoglycoprotein receptors (ASGPR), that recognize different sugar moieties such as lactose, galactose or mannose, allowing for liver-specific delivery [193, 194]. Jeong et al. [181] have prepared paclitaxel-loaded galactose-PEG-b-PBLG micelles, and showed a greater in vitro uptake and cytotoxicity of the micelles in an ASGPR-expressing cancer cell line compared to an analogous non-ASGPR expressing cell line. Alternatively, delivery system having lactose attached to the surface of PICM for siRNA delivery exhibited gene silencing of firefly luciferase expression in HuH-7 cells expressing ASGPR that was comparable to cationic liposomes (oligofectamine) [189].

16.10 Conclusions

Micelles are a promising drug delivery vehicle for drugs and genetic materials. These core-shell self-assemblies can also be tailored to increase the solubility of poorly water-soluble drugs just as protect labile hydrophilic drugs from premature degradation. Because of their nanometer size and hydrated outer layer, micelles can prolong the circulation time of an encapsulated drug and passively accumulate at tumor sites, thereby reducing its systemic toxicity and enhancing its efficacy. Micelles that actively target tissues can also be prepared by utilizing stimuli-responsive components or by attaching recognition groups at their surface. It is also important that the polymeric carriers need to be stable and retain the encapsulated drug long enough for any of these applications to be achievable. To meet this purpose, both the thermodynamic stability and kinetic stability of the micelles can be improved by varying the nature of the hydrophobic block, increasing the hydrophobic/hydrophilic balance, increasing the hydrophobic block length or by accommodating hydrophobic molecules in the core. In addition, micelle stabilization can also be achieved by cross-linking either the core or the corona of preformed micelles, by preparing crystalline micelles or by

designing intrinsically stable unimolecular polymeric micelles (UPM). The parameters affecting micelle stability, however, need to be carefully optimized with respect to their influence on the extent of drug solubilization and drug release kinetics. Much progress has been achieved to modulate the stability and stimuli responsiveness of PM in vitro, while, these strategies still remain to be tested in vivo to demonstrate real control over the pharmacological properties of the encapsulated drugs. With the structural requirements for micelle stability and drug release are still under conflict, future work should focus on the development and clinical application of multifunctional micelles capable of delivering drugs at target sites in a controlled/triggered fashion.

References

1. Zana R (2005) Dynamics of surfactant self-assemblies: micelles, microemulsions, vesicles and lyotropic phases. CRC press, Florida
2. Sinko PJ, Allen LV Jr, Popovich NG, Ansel HC (2006) Martin's physical pharmacy and pharmaceutical sciences, 5th edn. Lippincott Williams & Wilkins, Philadelphia
3. Adams ML, Lavasanifar A, Kwon GS (2003) Amphiphilic block copolymers for drug delivery. J Pharm Sci 92:1343–1355. doi:10.1002/jps.10397
4. Moroi Y (1992) Micelles: theoretical and applied aspects. Springer, New York
5. Tuzar Z, Kratochvil P (1976) Block and graft copolymer micelles in solution. Adv Colloid Interface Sci 6:201–232. doi:10.1016/0001-8686(76)80009-7
6. Hickok RS, Wedge SA, Hansen AL, Morris KF, Billiot FH, Warner IM (2002) Pulsed field gradient NMR investigation of solubilization equilibria in amino acid and dipeptide terminated micellar and polymeric surfactant solutions. Magn Reson Chem 40:755–761. doi:10.1002/mrc.1099
7. Jones M, Leroux J (1999) Polymeric micelles—a new generation of colloidal drug carriers. Eur J Pharm Biopharm 48:101–111. doi:10.1016/S0939-6411(99)00039-9
8. Erhardt R, Böker A, Zettl H, Kaya H, Pyckhout-Hintzen W, Krausch G, Abetz V, Müller AH (2001) Janus micelles. Macromolecules 34:1069–1075. doi:10.1021/ma000670p
9. Riess G, Hurtrez G, Bohadur P (1985) Block copolymers. Wiley-Interscience, Encyclopedia of Polymer Science and Engineering 2: 324–434
10. Xu J, Ge Z, Zhu Z, Luo S, Liu H, Liu S (2006) Synthesis and micellization properties of double hydrophilic A_2BA_2 and A_4BA_4 non-linear block copolymers. Macromolecules 39: 8178–8185. doi:10.1021/ma061934w
11. Bouchemal K, Agnely F, Koffi A, Ponchel G (2009) A concise analysis of the effect of temperature and propanediol-1, 2 on Pluronic F127 micellization using isothermal titration microcalorimetry. J Colloid Interface Sci 338:169–176. doi:10.1016/j.jcis.2009.05.075
12. Ranger M, Jones MC, Yessine MA, Leroux JC (2001) From well-defined diblock copolymers prepared by a versatile atom transfer radical polymerization method to supramolecular assemblies. J Polym Sci A Polym Chem 39:3861–3874. doi:10.1002/pola.10029
13. Zhang J, Ma PX (2009) Host-guest interaction mediated polymeric core-shell assemblies: versatile nanocarriers for drug delivery. Angew Chem Int Ed Engl 48:964. doi:10.1002/anie.200804135
14. Liu YL, Lin GC, Wu CS (2008) Preparation of polysulfone-g-poly (N-isopropylacrylamide) graft copolymers through atom transfer radical polymerization and formation of temperature-responsive nanoparticles. J Polym Sci A Polym Chem 46:4756–4765. doi:10.1002/pola.22809
15. Peng X, Zhang L (2007) Formation and morphologies of novel self-assembled micelles from chitosan derivatives. Langmuir 23:10493–10498

16. Kriz J, Pleštil J, Tuzar Z, Pospíšil H, Brus J, Jakeš J, Masar B, Vlcek P, Doskocilova D (1999) Interface affected polymer dynamics: NMR, SANS, and DLS study of the influence of shell-core interactions on the core chain mobility of poly (2-ethylhexyl acrylate)-block-poly (acrylic acid) micelles in water. Macromolecules 32:397–410. doi:10.1021/ma9809334
17. Procházka K, Martin TJ, Munk P, Webber SE (1996) Polyelectrolyte poly (tert-butyl acrylate)-block-poly (2-vinylpyridine) micelles in aqueous media. Macromolecules 29:6518–6525. doi:10.1021/ma960630e
18. Geng Y, Discher DE (2005) Hydrolytic degradation of poly (ethylene oxide)-block-polycaprolactone worm micelles. J Am Chem Soc 127:12780–12781
19. Hu Y, Zhang L, Cao Y, Ge H, Jiang X, Yang C (2004) Degradation behavior of poly (ε-caprolactone)-b-poly (ethylene glycol)-b-poly (ε-caprolactone) micelles in aqueous solution. Biomacromolecules 5:1756–1762
20. Xu B, Yuan J, Ding T, Gao Q (2010) Amphiphilic biodegradable poly (ε-caprolactone)-poly (ethylene glycol)-poly (ε-caprolactone) triblock copolymers: synthesis, characterization and their use as drug carriers for folic acid. Polym Bull 64:537–551. doi:10.1007/s00289-009-0157-5
21. Lee SC, Kim KJ, Jeong Y-K, Chang JH, Choi J (2005) pH-Induced reversible complexation of poly (ethylene glycol) and poly (ε-caprolactone)-b-poly (methacrylic acid) copolymer micelles. Macromolecules 38:9291–9297. doi:10.1021/ma051380h
22. Danhier F, Magotteaux N, Ucakar B, Lecouturier N, Brewster M, Préat V (2009) Novel self-assembling PEG-p-(CL-< i> co-TMC) polymeric micelles as safe and effective delivery system for Paclitaxel. Eur J Pharm Biopharm 73:230–238
23. Arimura H, Ohya Y, Ouchi T (2004) The formation of biodegradable polymeric micelles from newly synthesized poly (aspartic acid)-block-polylactide AB-type diblock copolymers. Macromol Rapid Commun 25:743–747
24. Alani AW, Bae Y, Rao DA, Kwon GS (2010) Polymeric micelles for the pH-dependent controlled, continuous low dose release of paclitaxel. Biomaterials 31:1765–1772
25. Huang CK, Lo CL, Chen HH, Hsiue GH (2007) Multifunctional micelles for cancer cell targeting, distribution imaging, and anticancer drug delivery. Adv Funct Mater 17:2291–2297. doi:10.1002/adfm.200600818
26. Hu F-Q, Liu L-N, Du Y-Z, Yuan H (2009) Synthesis and antitumor activity of doxorubicin conjugated stearic acid-< i> g-chitosan oligosaccharide polymeric micelles. Biomaterials 30:6955–6963
27. Webber S (1998) Polymer micelles: an example of self-assembling polymers. J Phys Chem B 102:2618–2626. doi:10.1021/jp980386o
28. Gohy JF (2005) Block copolymer micelles. Adv Polym Sci 190:65–136. doi:10.1007/12_048
29. Jette KK, Law D, Schmitt EA, Kwon GS (2004) Preparation and drug loading of poly (ethylene glycol)-block-poly (ε-caprolactone) micelles through the evaporation of a cosolvent azeotrope. Pharm Res 21:1184–1191. doi:10.1023/B:PHAM.0000033005.25698.9c
30. Fournier E, Dufresne MH, Smith DC, Ranger M, Leroux JC (2004) A novel one-step drug-loading procedure for water-soluble amphiphilic nanocarriers. Pharm Res 21:962–968. doi:10.1023/B:PHAM.0000029284.40637.69
31. La SB, Okano T, Kataoka K (1996) Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated poly(ethylene oxide)-poly(beta-benzyl L-aspartate) block copolymer micelles. J Pharm Sci 85:85–90. doi:10.1021/js950204r
32. Lipinski CA (2000) Drug-like properties and the causes of poor solubility and poor permeability. J Pharmacol Toxicol Methods 44:235–249. doi:10.1016/S1056-8719(00)00107-6
33. Attwood D, Booth C, Yeates SG, Chaibundit C, Ricardo NM (2007) Block copolymers for drug solubilisation: relative hydrophobicities of polyether and polyester micelle-core-forming blocks. Int J Pharm 345:35–41. doi:10.1016/j.ijpharm.2007.07.039
34. Hildebrand JH (1949) A critique of the theory of solubility of non-electrolytes. Chem Rev 44:37–45. doi:10.1021/cr60137a003
35. Flory PJ (1953) Principles of polymer chemistry. Cornell University Press, New York

36. Liu RR, Forrest ML, Kwon GS (2008) 13 Micellization and drug solubility enhancement part II: polymeric micelles. Water-Insoluble Drug Formulation, 307
37. Letchford K, Liggins R, Burt H (2008) Solubilization of hydrophobic drugs by methoxy poly(ethylene glycol)-block-polycaprolactone diblock copolymer micelles: theoretical and experimental data and correlations. J Pharm Sci 97:1179–1190. doi:10.1002/jps.21037
38. Ooya T, Sang CL, Kang MH, Park K (2006) Hydrotropic nanocarriers for poorly soluble drugs. In: Mozafari MR (ed) Nanocarrier technologies. Springer, Netherlands
39. Coffman RE, Kildsig DO (1996) Hydrotropic solubilization—mechanistic studies. Pharm Res 13:1460–1463. doi:10.1023/A:1016011125302
40. Bauduin P, Renoncourt A, Kopf A, Touraud D, Kunz W (2005) Unified concept of solubilization in water by hydrotropes and cosolvents. Langmuir 21:6769–6775. doi:10.1021/la050554l
41. Charman W, Lai C, Craik D, Finnin B, Reed B (1993) Self-association of nicotinamide in aqueous-solution: NMR studies of nicotinamide and the mono-and di-methyl-substituted amide analogs. Aust J Chem 46:377–385. doi:10.1071/CH9930377
42. Landauer J, McConnell H (1952) A study of molecular complexes formed by aniline and aromatic nitrohydrocarbons1, 2. J Am Chem Soc 74:1221–1224. doi:10.1021/ja01125a025
43. Quina FH, Alonso EO, Farah JP (1995) Incorporation of nonionic solutes into aqueous micelles: a linear solvation free energy relationship analysis. J Phys Chem 99:11708–11714. doi:10.1021/j100030a014
44. Rasool AA, Hussain AA, Dittert LW (1991) Solubility enhancement of some water-insoluble drugs in the presence of nicotinamide and related compounds. J Pharm Sci 80:387–393. doi:10.1002/jps.2600800422
45. Sanghvi R, Evans D, Yalkowsky SH (2007) Stacking complexation by nicotinamide: a useful way of enhancing drug solubility. Int J Pharm 336:35–41. doi:10.1016/j.ijpharm.2006.11.025
46. Calderara F, Hruska Z, Hurtrez G, Lerch J, Nugay T, Riess G (1994) Investigation of polystyrene-poly (ethylene oxide) block copolymer micelle formation in organic and aqueous solutions by nonradiative energy transfer experiments. Macromolecules 27:1210–1215. doi:10.1021/ma00083a020
47. Wang Y, Kausch CM, Chun M, Quirk RP, Mattice WL (1995) Exchange of chains between micelles of labeled polystyrene-block-poly (oxyethylene) as monitored by nonradiative singlet energy transfer. Macromolecules 28:904–911. doi:10.1021/ma00108a016
48. Wilhelm M, Zhao CL, Wang Y, Xu R, Winnik MA, Mura JL, Riess G, Croucher MD (1991) Poly (styrene-ethylene oxide) block copolymer micelle formation in water: a fluorescence probe study. Macromolecules 24:1033–1040. doi:10.1021/ma00005a010
49. Desjardins A, Eisenberg A (1991) Colloidal properties of block ionomers. 1. Characterization of reverse micelles of styrene-b-metal methacrylate diblocks by size-exclusion chromatography. Macromolecules 24:5779–5790
50. Trivedi R, Kompella UB (2010) Nanomicellar formulations for sustained drug delivery: strategies and underlying principles. Nanomedicine (Lond) 5:485–505. doi:10.2217/nnm.10.10
51. Nishiyama N, Kataoka K (2006) Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol Ther 112:630–648. doi:10.1016/j.pharmthera.2006.05.006
52. Thurmond KB, Kowalewski T, Wooley KL (1996) Water-soluble knedel-like structures: the preparation of shell-cross-linked small particles. J Am Chem Soc 118:7239–7240. doi:10.1021/ja961299h
53. Li T, Ning F, Xie J, Chen D, Jiang M (2002) Preparation and morphologies of shell cross-linked micelles based on commercial poly (styrene-block-ethylene-co-butene-block-styrene). Polym J 34:529–533. doi:10.1295/polymj.34.529
54. Zhang JX, Yan MQ, Li XH, Qiu LY, Li XD, Li XJ, Jin Y, Zhu KJ (2007) Local delivery of indomethacin to arthritis-bearing rats through polymeric micelles based on amphiphilic polyphosphazenes. Pharm Res 24:1944–1953. doi:10.1007/s11095-007-9322-4
55. Kakizawa Y, Harada A, Kataoka K (1999) Environment-sensitive stabilization of core-shell structured polyion complex micelle by reversible cross-linking of the core through disulfide bond. J Am Chem Soc 121:11247–11248. doi:10.1021/ja993057y

56. Hu X, Chen X, Wei J, Liu S, Jing X (2009) Core crosslinking of biodegradable block copolymer micelles based on poly(ester carbonate). Macromol Biosci 9:456–463. doi:10.1002/mabi.200800158
57. Rapoport N (1999) Stabilization and activation of Pluronic micelles for tumor-targeted drug delivery. Colloids Surf B: Biointerfaces 16:93–111. doi:10.1016/S0927-7765(99)00063-6
58. Bogdanov AA Jr, Mt L, Weissleder R (1999) Approaches and agents for imaging the vascular system. Adv Drug Deliv Rev 37:279–293. doi:10.1016/S0169-409X(98)00098-2
59. Yokoyama M, Okano T, Sakurai Y, Ekimoto H, Shibazaki C, Kataoka K (1991) Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. Cancer Res 51:3229–3236
60. Lebduskova P, Kotek J, Hermann P, Vander Elst L, Muller RN, Lukes I, Peters JA (2004) A gadolinium(III) complex of a carboxylic-phosphorus acid derivative of diethylenetriamine covalently bound to inulin, a potential macromolecular MRI contrast agent. Bioconjug Chem 15:881–889. doi:10.1021/bc049966g
61. Caravan P, Ellison JJ, McMurry TJ, Lauffer RB (1999) Gadolinium (III) chelates as MRI contrast agents: structure, dynamics, and applications. Chem Rev 99:2293–2352. doi:10.1021/cr980440x
62. Pathak AP, Gimi B, Glunde K, Ackerstaff E, Artemov D, Bhujwalla ZM (2004) Molecular and functional imaging of cancer: advances in MRI and MRS. Methods Enzymol 386:3–60
63. Jeong JH, Park TG (2001) Novel polymer-DNA hybrid polymeric micelles composed of hydrophobic poly (D, L-lactic-co-glycolic acid) and hydrophilic oligonucleotides. Bioconjug Chem 12:917–923
64. Jeong YI, Cheon JB, Kim SH, Nah JW, Lee YM, Sung YK, Akaike T, Cho CS (1998) Clonazepam release from core-shell type nanoparticles in vitro. J Control Release 51:169–178. doi:10.1016/S0168-3659(97)00163-6
65. Teng Y, Morrison M, Munk P, Webber S, Procházka K (1998) Release kinetics studies of aromatic molecules into water from block polymer micelles. Macromolecules 31:3578–3587. doi:10.1021/ma971721u
66. Rösler A, Vandermeulen GW, Klok H-A (2012) Advanced drug delivery devices via self-assembly of amphiphilic block copolymers. Adv Drug Deliv Rev 64:270–279. doi:10.1016/j.addr.2012.09.026
67. Shi B, Fang C, You MX, Zhang Y, Fu S, Pei Y (2005) Stealth MePEG-PCL micelles: effects of polymer composition on micelle physicochemical characteristics, in vitro drug release, in vivo pharmacokinetics in rats and biodistribution in S180 tumor bearing mice. Colloid Polym Sci 283:954–967. doi:10.1007/s00396-004-1243-8
68. Savic R, Azzam T, Eisenberg A, Maysinger D (2006) Assessment of the integrity of poly (caprolactone)-b-poly(ethylene oxide) micelles under biological conditions: a fluorogenic-based approach. Langmuir 22:3570–3578. doi:10.1021/la0531998
69. Burt HM, Zhang X, Toleikis P, Embree L, Hunter WL (1999) Development of copolymers of poly (D, L-lactide) and methoxypolyethylene glycol as micellar carriers of paclitaxel. Colloids Surf B: Biointerfaces 16:161–171. doi:10.1016/S0927-7765(99)00067-3
70. Chen H, Kim S, He W, Wang H, Low PS, Park K, Cheng JX (2008) Fast release of lipophilic agents from circulating PEG-PDLLA micelles revealed by in vivo forster resonance energy transfer imaging. Langmuir 24:5213–5217. doi:10.1021/la703570m
71. Gao Z, Fain HD, Rapoport N (2004) Ultrasound-enhanced tumor targeting of polymeric micellar drug carriers. Mol Pharm 1:317–330. doi:10.1021/mp049958h
72. Gao ZG, Fain HD, Rapoport N (2005) Controlled and targeted tumor chemotherapy by micellar-encapsulated drug and ultrasound. J Control Release 102:203–222. doi:10.1016/j.jconrel.2004.09.021
73. Tannock IF, Rotin D (1989) Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 49:4373–4384
74. Schild HG, Tirrell DA (1991) Microheterogeneous solutions of amphiphilic copolymers of N-isopropylacrylamide. An investigation via fluorescence methods. Langmuir 7:1319–1324
75. Gillies ER, Frechet JM (2005) pH-Responsive copolymer assemblies for controlled release of doxorubicin. Bioconjug Chem 16:361–368. doi:10.1021/bc049851c

76. Gillies ER, Jonsson TB, Frechet JM (2004) Stimuli-responsive supramolecular assemblies of linear-dendritic copolymers. J Am Chem Soc 126:11936–11943. doi:10.1021/ja0463738
77. Chung J, Yokoyama M, Yamato M, Aoyagi T, Sakurai Y, Okano T (1999) Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly (*N*-isopropylacrylamide) and poly (butylmethacrylate). J Control Release 62:115–127
78. Chung J, Yokoyama M, Aoyagi T, Sakurai Y, Okano T (1998) Effect of molecular architecture of hydrophobically modified poly (*N*-isopropylacrylamide) on the formation of thermo-responsive core-shell micellar drug carriers. J Control Release 53:119–130. doi:10.1016/S0168-3659(97)00244-7
79. Chen G, Hoffman AS (1995) Graft copolymers that exhibit temperature-induced phase transitions over a wide range of pH. Nature 373:49–52
80. Nakayama M, Okano T (2005) Polymer terminal group effects on properties of thermoresponsive polymeric micelles with controlled outer-shell chain lengths. Biomacromolecules 6:2320–2327
81. Taillefer J, Jones MC, Brasseur N, van Lier JE, Leroux JC (2000) Preparation and characterization of pH-responsive polymeric micelles for the delivery of photosensitizing anticancer drugs. J Pharm Sci 89:52–62. doi:10.1002/(SICI)1520-6017(200001)89:1<52::AID-JPS6>3.0.CO;2-D
82. Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420:860–867. doi:10.1038/nature01322
83. Rehor A, Schmoekel H, Tirelli N, Hubbell JA (2008) Functionalization of polysulfide nanoparticles and their performance as circulating carriers. Biomaterials 29:1958–1966. doi:10.1016/j.biomaterials.2007.12.035
84. Rehor A, Hubbell JA, Tirelli N (2005) Oxidation-sensitive polymeric nanoparticles. Langmuir 21:411–417. doi:10.1021/la0478043
85. Kim SH, Jeong JH, Lee SH, Kim SW, Park TG (2006) PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. J Control Release 116:123–129
86. Lee SH, Kim SH, Park TG (2007) Intracellular siRNA delivery system using polyelectrolyte complex micelles prepared from VEGF siRNA-PEG conjugate and cationic fusogenic peptide. Biochem Biophys Res Commun 357:511–516. doi:10.1016/j.bbrc.2007.03.185
87. Allen C, Yu Y, Eisenberg A, Maysinger D (1999) Cellular internalization of PCL_{20}-b-PEO_{44} block copolymer micelles. BBA Biomembranes 1421:32–38. doi:10.1016/S0005-2736(99)00108-X
88. Poon GM, Gariepy J (2007) Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. Biochem Soc Trans 35:788–793. doi:10.1042/BST0350788
89. Miller DW, Batrakova EV, Waltner TO, Alakhov V, Kabanov AV (1997) Interactions of pluronic block copolymers with brain microvessel endothelial cells: evidence of two potential pathways for drug absorption. Bioconjug Chem 8:649–657. doi:10.1021/bc970118d
90. Rapoport N, Marin A, Luo Y, Prestwich GD, Muniruzzaman MD (2002) Intracellular uptake and trafficking of Pluronic micelles in drug-sensitive and MDR cells: effect on the intracellular drug localization. J Pharm Sci 91:157–170. doi:10.1002/jps.10006
91. Zastre JA, Jackson JK, Wong W, Burt HM (2008) P-glycoprotein efflux inhibition by amphiphilic diblock copolymers: relationship between copolymer concentration and substrate hydrophobicity. Mol Pharm 5:643–653. doi:10.1021/mp7001347
92. Mikhail AS, Allen C (2009) Block copolymer micelles for delivery of cancer therapy: transport at the whole body, tissue and cellular levels. J Control Release 138:214–223. doi:10.1016/j.jconrel.2009.04.010
93. Park YJ, Lee JY, Chang YS, Jeong JM, Chung JK, Lee MC, Park KB, Lee SJ (2002) Radioisotope carrying polyethylene oxide–polycaprolactone copolymer micelles for targetable bone imaging. Biomaterials 23:873–879. doi:10.1016/s0142-9612(01)00196-x
94. Liu J, Zeng F, Allen C (2007) In vivo fate of unimers and micelles of a poly(ethylene glycol)-block-poly(caprolactone) copolymer in mice following intravenous administration. Eur J Pharm Biopharm 65:309–319. doi:10.1016/j.ejpb.2006.11.010

95. Lin WJ, Chen YC, Lin CC, Chen CF, Chen JW (2006) Characterization of pegylated copolymeric micelles and in vivo pharmacokinetics and biodistribution studies. J Biomed Mater Res B Appl Biomater 77:188–194. doi:10.1002/jbm.b.30418
96. Aliabadi HM, Brocks DR, Lavasanifar A (2005) Polymeric micelles for the solubilization and delivery of cyclosporine A: pharmacokinetics and biodistribution. Biomaterials 26:7251–7259. doi:10.1016/j.biomaterials.2005.05.042
97. Novakova K, Laznicek M, Rypacek F, Machova L (2003) Pharmacokinetics and distribution of 125I-PLA-b-PEO block copolymers in rats. Pharm Dev Technol 8:153–161. doi:10.1081/PDT-120018484
98. Kim SC, Kim DW, Shim YH, Bang JS, Oh HS, Wan Kim S, Seo MH (2001) In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. J Control Release 72:191–202. doi:10.1016/S0168-3659(01)00275-9
99. Yamamoto Y, Nagasaki Y, Kato Y, Sugiyama Y, Kataoka K (2001) Long-circulating poly(ethylene glycol)–poly(d, l-lactide) block copolymer micelles with modulated surface charge. J Control Release 77:27–38. doi:10.1016/s0168-3659(01)00451-5
100. Hu Z, Luo F, Pan Y, Hou C, Ren L, Chen J, Wang J, Zhang Y (2008) Arg-Gly-Asp (RGD) peptide conjugated poly(lactic acid)-poly(ethylene oxide) micelle for targeted drug delivery. J Biomed Mater Res A 85:797–807. doi:10.1002/jbm.a.31615
101. Kawano K, Watanabe M, Yamamoto T, Yokoyama M, Opanasopit P, Okano T, Maitani Y (2006) Enhanced antitumor effect of camptothecin loaded in long-circulating polymeric micelles. J Control Release 112:329–332. doi:10.1016/j.jconrel.2006.03.012
102. Kawakami S, Opanasopit P, Yokoyama M, Chansri N, Yamamoto T, Okano T, Yamashita F, Hashida M (2005) Biodistribution characteristics of all-trans retinoic acid incorporated in liposomes and polymeric micelles following intravenous administration. J Pharm Sci 94:2606–2615. doi:10.1002/jps.20487
103. Batrakova EV, Li S, Li Y, Alakhov VY, Elmquist WF, Kabanov AV (2004) Distribution kinetics of a micelle-forming block copolymer Pluronic P85. J Control Release 100:389–397. doi:10.1016/j.jconrel.2004.09.002
104. Wang Y, Li Y, Zhang L, Fang X (2008) Pharmacokinetics and biodistribution of paclitaxel-loaded pluronic P105 polymeric micelles. Arch Pharm Res 31:530–538. doi:10.1007/s12272-001-1189-2
105. Le Garrec D, Gori S, Luo L, Lessard D, Smith D, Yessine M-A, Ranger M, Leroux J-C (2004) Poly (*N*-vinylpyrrolidone)-block-poly (d, l-lactide) as a new polymeric solubilizer for hydrophobic anticancer drugs: in vitro and in vivo evaluation. J Control Release 99:83–101. doi:10.1016/j.jconrel.2004.06.018
106. Bae Y, Nishiyama N, Kataoka K (2007) In vivo antitumor activity of the folate-conjugated pH-sensitive polymeric micelle selectively releasing adriamycin in the intracellular acidic compartments. Bioconjug Chem 18:1131–1139. doi:10.1021/bc060401p
107. Tian HY, Deng C, Lin H, Sun J, Deng M, Chen X, Jing X (2005) Biodegradable cationic PEG-PEI-PBLG hyperbranched block copolymer: synthesis and micelle characterization. Biomaterials 26:4209–4217. doi:10.1016/j.biomaterials.2004.11.002
108. Rijcken CJ, Snel CJ, Schiffelers RM, van Nostrum CF, Hennink WE (2007) Hydrolysable core-crosslinked thermosensitive polymeric micelles: Synthesis, characterisation and in vivo studies. Biomaterials 28:5581–5593. doi:10.1016/j.biomaterials.2007.08.047
109. Yokoyama M, Kwon GS, Okano T, Sakurai Y, Seto T, Kataoka K (1992) Preparation of micelle-forming polymer-drug conjugates. Bioconjug Chem 3:295–301. doi:10.1021/bc00016a007
110. Hoes C, Potman W, Van Heeswijk W, Mud J, De Grooth B, Greve J, Feijen J (1985) Optimization of macromolecular prodrugs of the antitumor antibiotic adriamycin. J Control Release 2:205–213. doi:10.1016/0168-3659(85)90046-X
111. Duncan R, Kopeckova-Rejmanova P, Strohalm J, Hume I, Cable HC, Pohl J, Lloyd JB, Kopecek J (1987) Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. I. Evaluation of daunomycin and puromycin conjugates in vitro. Br J Cancer 55:165–174

112. Endo N, Umemoto N, Kato Y, Takeda Y, Hara T (1987) A novel covalent modification of antibodies at their amino groups with retention of antigen-binding activity. J Immunol Methods 104:253–258. doi:10.1016/0022-1759(87)90512-6
113. Zunino F, Pratesi G, Micheloni A (1989) Poly (carboxylic acid) polymers as carriers for anthracyclines. J Control Release 10:65–73. doi:10.1016/0168-3659(89)90018-7
114. Harada A, Kataoka K (1998) Novel polyion complex micelles entrapping enzyme molecules in the core: preparation of narrowly-distributed micelles from lysozyme and poly (ethylene glycol)-poly (aspartic acid) block copolymer in aqueous medium. Macromolecules 31:288–294. doi:10.1021/ma971277v
115. Kataoka K, Togawa H, Harada A, Yasugi K, Matsumoto T, Katayose S (1996) Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. Macromolecules 29:8556–8557. doi:10.1021/ma961217+
116. Yokoyama M, Okano T, Sakurai Y, Suwa S, Kataoka K (1996) Introduction of cisplatin into polymeric micelle. J Control Release 39:351–356. doi:10.1016/0168-3659(95)00165-4
117. Nishiyama N, Okazaki S, Cabral H, Miyamoto M, Kato Y, Sugiyama Y, Nishio K, Matsumura Y, Kataoka K (2003) Novel cisplatin-incorporated polymeric micelles can eradicate solid tumors in mice. Cancer Res 63:8977–8983
118. Nakamura E, Makino K, Okano T, Yamamoto T, Yokoyama M (2006) A polymeric micelle MRI contrast agent with changeable relaxivity. J Control Release 114:325–333. doi:10.1016/j.jconrel.2006.05.030
119. Shiraishi K, Kawano K, Maitani Y, Yokoyama M (2010) Polyion complex micelle MRI contrast agents from poly(ethylene glycol)-b-poly(l-lysine) block copolymers having Gd-DOTA; preparations and their control of T_1-relaxivities and blood circulation characteristics. J Control Release 148:160–167. doi:10.1016/j.jconrel.2010.08.018
120. Clark SL, Crowley AJ, Schmidt PG, Donoghue AR, Piché CA (2004) Long-term delivery of ivermectin by use of poly (D, L-lactic-co-glycolic) acid microparticles in dogs. Am J Vet Res 65:752–757. doi:10.2460/ajvr.2004.65.752
121. Sartor O (2003) Eligard: leuprolide acetate in a novel sustained-release delivery system. Urology 61:25–31. doi:10.1016/S0090-4295(02)02396-8
122. Singh SR, Grossniklaus HE, Kang SJ, Edelhauser HF, Ambati BK, Kompella UB (2009) Intravenous transferrin, RGD peptide and dual-targeted nanoparticles enhance anti-VEGF intraceptor gene delivery to laser-induced CNV. Gene Ther 16:645–659. doi:10.1038/gt.2008.185
123. Raghuvanshi RS, Singh O, Panda AK (2001) Formulation and characterization of immunoreactive tetanus toxoid biodegradable polymer particles. Drug Deliv 8:99–106. doi:10.1080/107175401750177089
124. Forrest ML, Yanez JA, Remsberg CM, Ohgami Y, Kwon GS, Davies NM (2008) Paclitaxel prodrugs with sustained release and high solubility in poly(ethylene glycol)-b-poly(epsilon-caprolactone) micelle nanocarriers: pharmacokinetic disposition, tolerability, and cytotoxicity. Pharm Res 25:194–206. doi:10.1007/s11095-007-9451-9
125. Zhang N, Guo SR, Li HQ, Liu L, Li ZH, Gu JR (2006) Synthesis of Three Types of Amphiphilic Poly (ethylene glycol)-block-Poly (sebacic anhydride) Copolymers and Studies of their Micellar Solutions. Macromol Chem Phys 207:1359–1367. doi:10.1002/macp.200600100
126. Gaber NN, Darwis Y, Peh KK, Tan YT (2006) Characterization of polymeric micelles for pulmonary delivery of beclomethasone dipropionate. J Nanosci Nanotechnol 6:3095–3101. doi:10.1166/jnn.2006.426
127. Lavasanifar A, Samuel J, Kwon GS (2002) The effect of fatty acid substitution on the in vitro release of amphotericin B from micelles composed of poly(ethylene oxide)-*block*-poly(*N*-hexyl stearate-l-aspartamide). J Control Release 79:165–172. doi:10.1016/S0168-3659(01)00537-5
128. Dong H, Li Y, Cai S, Zhuo R, Zhang X, Liu L (2008) A facile one-pot construction of supramolecular polymer micelles from alpha-cyclodextrin and poly(epsilon-caprolactone). Angew Chem Int Ed Engl 47:5573–5576. doi:10.1002/anie.200800952

129. Yang X, Zhu B, Dong T, Pan P, Shuai X, Inoue Y (2008) Interactions between an anticancer drug and polymeric micelles based on biodegradable polyesters. Macromol Biosci 8:1116–1125. doi:10.1002/mabi.200800085
130. Yokoyama M, Okano T (1996) Targetable drug carriers: present status and a future perspective. Adv Drug Deliv Rev 21:77–80. doi:10.1016/S0169-409X(96)00439-5
131. Sugiyama Y (1996) Importance of pharmacokinetic considerations in the development of drug delivery systems. Adv Drug Deliv Rev 19:333–334. doi:10.1016/0169-409X(96)00007-5
132. Litzinger DC, Buiting AM, van Rooijen N, Huang L (1994) Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. Biochim Biophys Acta 1190:99–107. doi:10.1016/0005-2736(94)90038-8
133. Takakura Y, Hashida M (1996) Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution. Pharm Res 13:820–831. doi:10.1023/A:1016084508097
134. Illum L, Davis S, Müller R, Mak E, West P (1987) The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilized with a blockcopolymer-poloxamine 908. Life Sci 40:367–374. doi:10.1016/0024-3205(87)90138-X
135. Yokoyama M, Inoue S, Kataoka K, Yui N, Sakurai Y (1987) Preparation of adriamycin-conjugated poly (ethylene glycol)-poly (aspartic acid) block copolymer. A new type of polymeric anticancer agent. Die Makromolekulare Chemie, Rapid Commun 8:431–435
136. Yokoyama M, Miyauchi M, Yamada N, Okano T, Sakurai Y, Kataoka K, Inoue S (1990) Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. Cancer Res 50:1693–1700
137. Yokoyama M, Okano T, Sakurai Y, Fukushima S, Okamoto K, Kataoka K (1999) Selective delivery of adiramycin to a solid tumor using a polymeric micelle carrier system. J Drug Target 7:171–186. doi:10.3109/10611869909085500
138. Shiraishi K, Kawano K, Minowa T, Maitani Y, Yokoyama M (2009) Preparation and in vivo imaging of PEG-poly(L-lysine)-based polymeric micelle MRI contrast agents. J Control Release 136:14–20. doi:10.1016/j.jconrel.2009.01.010
139. Engin K, Leeper DB, Cater JR, Thistlethwaite AJ, Tupchong L, McFarlane JD (1995) Extracellular pH distribution in human tumours. Int J Hyperthermia 11:211–216. doi:10.3109/02656739509022457
140. Helmlinger G, Yuan F, Dellian M, Jain RK (1997) Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3:177–182. doi:10.1038/nm0297-177
141. Bae Y, Diezi TA, Zhao A, Kwon GS (2007) Mixed polymeric micelles for combination cancer chemotherapy through the concurrent delivery of multiple chemotherapeutic agents. J Control Release 122:324–330. doi:10.1016/j.jconrel.2007.05.038
142. Chung J, Yokoyama M, Yamato M, Aoyagi T, Sakurai Y, Okano T (1999) Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly (*N*-isopropylacrylamide) and poly (butylmethacrylate). J Control Release 62:115–127. doi:10.1016/S0168-3659(99)00029-2
143. Jaeghere F, Allemann E, Feijen J, Kissel T, Doelker E, Gurny R (2000) Cellular uptake of PEO surface-modified nanoparticles: evaluation of nanoparticles made of PLA: PEO diblock and triblock copolymers. J Drug Target 8:143–153. doi:10.3109/10611860008996860
144. Ross JF, Chaudhuri PK, Ratnam M (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. Cancer 73:2432–2443
145. Ulbrich K, Etrych T, Chytil P, Jelinkova M, Rihova B (2003) HPMA copolymers with pH-controlled release of doxorubicin: in vitro cytotoxicity and in vivo antitumor activity. J Control Release 87:33–47. doi:10.1016/S0168-3659(02)00348-6
146. Merdan T, Callahan J, Petersen H, Kunath K, Bakowsky U, Kopečková P, Kissel T, Kopecek J (2003) Pegylated polyethylenimine-Fab′ antibody fragment conjugates for targeted

gene delivery to human ovarian carcinoma cells. Bioconjug Chem 14:989–996, 10.1021/bc0340767
147. Vinogradov S, Batrakova E, Li S, Kabanov A (1999) Polyion complex micelles with protein-modified corona for receptor-mediated delivery of oligonucleotides into cells. Bioconjug Chem 10:851–860. doi:10.1021/bc990037c
148. Nah J-W, Yu L, S-o H, Ahn C-H, Kim SW (2002) Artery wall binding peptide-poly(ethylene glycol)-*grafted*-poly(l-lysine)-based gene delivery to artery wall cells. J Control Release 78:273–284. doi:10.1016/S0168-3659(01)00499-0
149. Farokhzad OC, Jon S, Khademhosseini A, Tran TN, Lavan DA, Langer R (2004) Nanoparticle-aptamer bioconjugates: a new approach for targeting prostate cancer cells. Cancer Res 64:7668–7672. doi:10.1158/0008-5472.CAN-04-2550
150. Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, Richie JP, Langer R (2006) Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo. Proc Natl Acad Sci U S A 103:6315–6320. doi:10.1073/pnas.0601755103
151. Lim DW, Yeom YI, Park TG (2000) Poly (DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. Bioconjug Chem 11:688–695. doi:10.1021/bc000014u
152. Yoo HS, Lee EA, Park TG (2002) Doxorubicin-conjugated biodegradable polymeric micelles having acid-cleavable linkages. J Control Release 82:17–27. doi:10.1016/S0168-3659(02)00088-3
153. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, Brampton M, Halbert G, Ranson M (2004) Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. Br J Cancer 90:2085–2091. doi:10.1038/sj.bjc.6601856
154. Alakhov V, Klinski E, Li S, Pietrzynski G, Venne A, Batrakova E, Bronitch T, Kabanov A (1999) Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials. Colloids Surf B: Biointerfaces 16:113–134
155. Matsumura Y, Hamaguchi T, Ura T, Muro K, Yamada Y, Shimada Y, Shirao K, Okusaka T, Ueno H, Ikeda M, Watanabe N (2004) Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. Br J Cancer 91:1775–1781. doi:10.1038/sj.bjc.6602204
156. Venne A, Li S, Mandeville R, Kabanov A, Alakhov V (1996) Hypersensitizing effect of pluronic L61 on cytotoxic activity, transport, and subcellular distribution of doxorubicin in multiple drug-resistant cells. Cancer Res 56:3626–3629
157. Yoo HS, Park TG (2004) Folate receptor targeted biodegradable polymeric doxorubicin micelles. J Control Release 96:273–283. doi:10.1016/j.jconrel.2004.02.003
158. Nasongkla N, Shuai X, Ai H, Weinberg BD, Pink J, Boothman DA, Gao J (2004) cRGD-functionalized polymer micelles for targeted doxorubicin delivery. Angew Chem Int Ed Engl 43:6323–6327. doi:10.1002/anie.200460800
159. Bae Y, Nishiyama N, Fukushima S, Koyama H, Yasuhiro M, Kataoka K (2005) Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. Bioconjug Chem 16:122–130. doi:10.1021/bc0498166
160. Kim TY, Kim DW, Chung JY, Shin SG, Kim SC, Heo DS, Kim NK, Bang YJ (2004) Phase I and pharmacokinetic study of Genexol-PM, a cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies. Clin Cancer Res 10:3708–3716. doi:10.1158/1078-0432.CCR-03-0655
161. Jeong YI, Seo SJ, Park IK, Lee HC, Kang IC, Akaike T, Cho CS (2005) Cellular recognition of paclitaxel-loaded polymeric nanoparticles composed of poly(gamma-benzyl L-glutamate) and poly(ethylene glycol) diblock copolymer endcapped with galactose moiety. Int J Pharm 296:151–161. doi:10.1016/j.ijpharm.2005.02.027
162. Torchilin VP, Lukyanov AN, Gao Z, Papahadjopoulos-Sternberg B (2003) Immunomicelles: targeted pharmaceutical carriers for poorly soluble drugs. Proc Natl Acad Sci U S A 100:6039–6044. doi:10.1073/pnas.0931428100

163. Elsabahy M, Perron ME, Bertrand N, Yu GE, Leroux JC (2007) Solubilization of docetaxel in poly(ethylene oxide)-block-poly(butylene/styrene oxide) micelles. Biomacromolecules 8:2250–2257. doi:10.1021/bm070226v
164. Taillefer J, Brasseur N, van Lier JE, Lenaerts V, Le Garrec D, Leroux JC (2001) In-vitro and in-vivo evaluation of pH-responsive polymeric micelles in a photodynamic cancer therapy model. J Pharm Pharmacol 53:155–166. doi:10.1211/0022357011775352
165. Le Garrec D, Taillefer J, Van Lier JE, Lenaerts V, Leroux JC (2002) Optimizing pH-responsive polymeric micelles for drug delivery in a cancer photodynamic therapy model. J Drug Target 10:429–437. doi:10.1080/1061186021000001887
166. Mizumura Y, Matsumura Y, Hamaguchi T, Nishiyama N, Kataoka K, Kawaguchi T, Hrushesky WJ, Moriyasu F, Kakizoe T (2001) Cisplatin-incorporated polymeric micelles eliminate nephrotoxicity, while maintaining antitumor activity. Cancer Sci 92:328–336. doi:10.1111/j.1349-7006.2001.tb01099.x
167. Nishiyama N, Kato Y, Sugiyama Y, Kataoka K (2001) Cisplatin-loaded polymer-metal complex micelle with time-modulated decaying property as a novel drug delivery system. Pharm Res 18:1035–1041. doi:10.1023/A:1010908916184
168. Nishiyama N, Yokoyama M, Aoyagi T, Okano T, Sakurai Y, Kataoka K (1999) Preparation and characterization of self-assembled polymer-metal complex micelle from cis-dichlorodiammineplatinum (II) and poly (ethylene glycol)-poly (α, β-aspartic acid) block copolymer in an aqueous medium. Langmuir 15:377–383. doi:10.1021/la980572l
169. Torchilin VP (2001) Structure and design of polymeric surfactant-based drug delivery systems. J Control Release 73:137–172. doi:10.1016/S0168-3659(01)00299-1
170. Adams ML, Andes DR, Kwon GS (2003) Amphotericin B encapsulated in micelles based on poly(ethylene oxide)-block-poly(L-amino acid) derivatives exerts reduced in vitro hemolysis but maintains potent in vivo antifungal activity. Biomacromolecules 4:750–757. doi:10.1021/bm0257614
171. Benahmed A, Ranger M, Leroux JC (2001) Novel polymeric micelles based on the amphiphilic diblock copolymer poly(N-vinyl-2-pyrrolidone)-block-poly(D, L-lactide). Pharm Res 18:323–328. doi:10.1023/A:1011054930439
172. Singh M (1999) Transferrin as a targeting ligand for liposomes and anticancer drugs. Curr Pharm Des 5:443–452
173. Qian ZM, Li H, Sun H, Ho K (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54:561–587
174. Allen TM (2002) Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2:750–763. doi:10.1038/nrc903
175. Torchilin VP, Lukyanov AN, Gao Z, Papahadjopoulos-Sternberg B (2003) Immunomicelles: targeted pharmaceutical carriers for poorly soluble drugs. Proc Natl Acad Sci 100: 6039–6044
176. Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346:818–822. doi:10.1038/346818a0
177. Lupold SE, Hicke BJ, Lin Y, Coffey DS (2002) Identification and characterization of nuclease-stabilized RNA molecules that bind human prostate cancer cells via the prostate-specific membrane antigen. Cancer Res 62:4029–4033
178. Elsabahy M, Zhang M, Gan S-M, Waldron KC, Leroux J-C (2008) Synthesis and enzymatic stability of PEGylated oligonucleotide duplexes and their self-assemblies with polyamidoamine dendrimers. Soft Matter 4:294–302. doi:10.1039/B714221H
179. Yessine MA, Dufresne MH, Meier C, Petereit HU, Leroux JC (2007) Proton-actuated membrane-destabilizing polyion complex micelles. Bioconjug Chem 18:1010–1014. doi:10.1021/bc060159m
180. Jeong JH, Kim SW, Park TG (2003) A new antisense oligonucleotide delivery system based on self-assembled ODN-PEG hybrid conjugate micelles. J Control Release 93:183–191. doi:10.1016/j.jconrel.2003.07.002
181. Jeong JH, Kim SH, Kim SW, Park TG (2005) Polyelectrolyte complex micelles composed of c-raf antisense oligodeoxynucleotide-poly(ethylene glycol) conjugate and poly(ethylenimine):

effect of systemic administration on tumor growth. Bioconjug Chem 16:1034–1037. doi:10.1021/bc0497315
182. Jeong JH, Kim SW, Park TG (2003) Novel intracellular delivery system of antisense oligonucleotide by self-assembled hybrid micelles composed of DNA/PEG conjugate and cationic fusogenic peptide. Bioconjug Chem 14:473–479. doi:10.1021/bc025632k
183. Kim SH, Jeong JH, Mok H, Lee SH, Kim SW, Park TG (2007) Folate receptor targeted delivery of polyelectrolyte complex micelles prepared from ODN-PEG-folate conjugate and cationic lipids. Biotechnol Prog 23:232–237. doi:10.1021/bp060243g
184. Harada A, Togawa H, Kataoka K (2001) Physicochemical properties and nuclease resistance of antisense-oligodeoxynucleotides entrapped in the core of polyion complex micelles composed of poly(ethylene glycol)-poly(L-lysine) block copolymers. Eur J Pharm Sci 13:35–42. doi:10.1016/S0928-0987(00)00205-0
185. Kakizawa Y, Harada A, Kataoka K (2001) Glutathione-sensitive stabilization of block copolymer micelles composed of antisense DNA and thiolated poly (ethylene glycol)-b lock-poly (l-lysine): a potential carrier for systemic delivery of antisense DNA. Biomacromolecules 2:491–497. doi:10.1021/bm000142l
186. Itaka K, Kanayama N, Nishiyama N, Jang WD, Yamasaki Y, Nakamura K, Kawaguchi H, Kataoka K (2004) Supramolecular nanocarrier of siRNA from PEG-based block catiomer carrying diamine side chain with distinctive pKa directed to enhance intracellular gene silencing. J Am Chem Soc 126:13612–13613. doi:10.1021/ja047174r
187. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ (2005) Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. Cancer Res 65:8984–8992. doi:10.1158/0008-5472.CAN-05-0565
188. Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, Molema G, Lu PY, Scaria PV, Woodle MC (2004) Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res 32:e149. doi:10.1093/nar/gnh140
189. Oishi M, Nagasaki Y, Itaka K, Nishiyama N, Kataoka K (2005) Lactosylated poly (ethylene glycol)-siRNA conjugate through acid-labile β-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. J Am Chem Soc 127:1624–1625. doi:10.1021/ja044941d
190. Woodle MC, Scaria P, Ganesh S, Subramanian K, Titmas R, Cheng C, Yang J, Pan Y, Weng K, Gu C, Torkelson S (2001) Sterically stabilized polyplex: ligand-mediated activity. J Control Release 74:309–311. doi:10.1016/S0168-3659(01)00339-X
191. Mizejewski GJ (1999) Role of integrins in cancer: survey of expression patterns. Proc Soc Exp Biol Med 222:124–138
192. Kamen BA, Smith AK (2004) A review of folate receptor alpha cycling and 5-methyltetrahydrofolate accumulation with an emphasis on cell models in vitro. Adv Drug Deliv Rev 56:1085–1097. doi:10.1016/j.addr.2004.01.002
193. Jule E, Nagasaki Y, Kataoka K (2003) Lactose-installed poly(ethylene glycol)-poly(d, l-lactide) block copolymer micelles exhibit fast-rate binding and high affinity toward a protein bed simulating a cell surface. A surface plasmon resonance study. Bioconjug Chem 14:177–186
194. Sugahara K, Togashi H, Takahashi K, Onodera Y, Sanjo M, Misawa K, Suzuki A, Adachi T, Ito J, Okumoto K (2003) Separate analysis of asialoglycoprotein receptors in the right and left hepatic lobes using 99mTc-GSA SPECT. Hepatology 38:1401–1409. doi:10.1016/j.hep.2003.09.031

Chapter 17
Dendritic Polymers in Targeted Drug Delivery

Sumati Bhatia and Rainer Haag

Abbreviations

8-MOP	8-Methoxypsoralen
Ab	Antibody
ABP	Azabisphosphonate
AF	Alexa Fluor
ApoE	Apolipoprotein E
BBB	Blood–brain barrier
BCSFB	Blood–cerebrospinal fluid barrier
BNCT	Boron neutron capture therapy
CMS	Core-multishell
CPT	Camptothecin
dPG	Dendritic polyglycerol
dPGS	Dendritic polyglycerol sulfate
DTX	Docetaxel
EGF	Epidermal growth factor
EPR	Enhanced permeability and retention effect
FA	Folic acid
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
G5	Generation 5
hPG	Hyperbranched polyglycerol

S. Bhatia • R. Haag (✉)
Institut für Chemie und Biochemie, Freie Universität Berlin,
Takustrasse 3, 14195 Berlin, Germany
e-mail: haag@chemie.fu-berlin.de

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_17

ICC	Indocarbocyanine
IDCC	Indodicarbocyanine
kDa	kiloDalton
LDL	Low density lipoproteins
Lf	Lactoferrin
LHRH	Luteinizing hormone-releasing hormone
MDR	Multiple drug resistance
MTX	Methotrexate
NLC	Nanostructured lipid carrier
OEI	Oligoethyleneimine
PABC	para-Aminobenzyloxycarbonyl
PAMAM	Polyamidoamine
PEHA	Pentaethylenehexamine
PEI	Poly(ethylene imine)
P-gp	P-Glycoprotein
PPI	Poly(propylene imine)
PSMA	Prostate-specific membrane antigen
RA	Rheumatoid arthritis
RGD	Arginylglycylaspartic acid
SC	Stratum corneum
SLN	Solid lipid nanoparticle
TAA	Tumor associated antigens
TAM	Tamoxifen
Tf	Transferrin

17.1 Introduction

Delivering chemotherapeutic agents specifically to the diseased tissues or cells in the body is the utmost challenge in the area of drug delivery. Targeted drug delivery not only helps to reduce the dose to be administered but also reduces the incidence of adverse effects associated with the chemotherapeutic agents. In past decades, dendritic polymers have emerged as promising scaffolds for targeted drug delivery. As a result, extensive research work has been published on dendritic nanostructures for the delivery of various drugs, e.g., camptothecin (CPT), taxol, cisplatin, methotrexate (MTX), etc. [1, 2]. Dendritic polymers have unique properties like a high degree of branching leading to multivalency, modifiable surface group functionalities, globular architecture, low polydispersity, and high molecular weight, which has made them attractive as scaffolds in the area of biomedical applications [3]. Dendritic polymers include perfect dendrimers, dendrons, dendronized polymers, and hyperbranched polymers. Perfect dendrimers and dendrons are monodisperse with nanometer dimensions (1–10 nm), low viscosity,

high solubility, multiple functionality at terminal groups, and biocompatibility. Hyperbranched and dendronized polymers can be in the size range of a nanometer to micrometer scale with a concomitant increase in the field of application.

A comparison of dendritic and linear polymers shows several advantages of dendritic polymer architectures for delivery applications. For example, a high degree of branching leading to multivalency in dendritic polymers can be used for the conjugation of different or similar drug molecules, targeting moieties, solubilizing modalities and/or imaging probes on the periphery of the same construct in a controlled fashion and makes them potential scaffolds for targeted drug delivery. In most of the cases, the linkage chemistry between the drug and polymer is of ester or amide type, but drug molecules have also been linked to the dendritic scaffolds through hydrazone, imine, carbamate, disulfide, carbazate bonds, and enzymatic cleavable peptide sequences. Each of the linkage modules has a different mechanism of cleavage that releases the drug molecule from a carrier scaffold. For example, cleavage of hydrazone, carbamate, and ester depends on the environmental pH changes and peptide and amide sequences require specific enzymes for degradation and subsequent release of active species. The globular shape of dendritic polymers can lead to the discovery of interesting effects due to their macromolecular architecture and could affect their chemical, mechanical, and biological properties [4]. Moreover, low polydispersity should provide a more reproducible pharmacokinetics behavior compared to linear polymers. Flexible branches of dendritic polymers can provide a tailored sanctuary containing voids that can be used for the encapsulation of drug molecules, where the drug molecules can stabilize themselves by assembling with the branching fragments through secondary interactions (hydrogen bonding, electrostatic interactions, dipole–dipole, and hydrophobic interactions) [5]. This leads to several advantages such as enhanced solubility of nonpolar drugs in aqueous media, minimization of non-specific interactions of the encapsulated drug molecules with plasma components, and an increased blood circulation half-life due to high molecular weight with an increasing number of branches [6, 7].

A number of dendritic backbones have been designed and developed to be water soluble and biocompatible for their use in drug delivery applications. For example, commercially available polyamidoamine (PAMAM) [8–10], poly(propylene imine) (PPI) [11], peptide based dendrimer polylysine [12], polyaryl ethers [13], polyester [14, 15], polyamide [16], polyglycerol (PG) [17, 18], and triazine dendrimers [19] have shown a higher efficacy in the field of polymer therapeutics (Fig. 17.1) [20]. Polyamidoamine (PAMAM) dendrimers are commercially available and are one of the most widely used dendritic scaffolds in biology [7–9, 21]. PPI dendritic polymers have been commercialized, but the presence of multiple cationic amine groups leads to significant toxicity [11]. Fréchet and Hawker developed polyaryl ether dendrimers that require, however, an extensive use of solubilizing groups at their periphery due to their poor water solubility [13]. More recently, glycerol based polyethers and polyesters have shown to be extremely biocompatible and suitable for drug delivery purposes [22, 23].

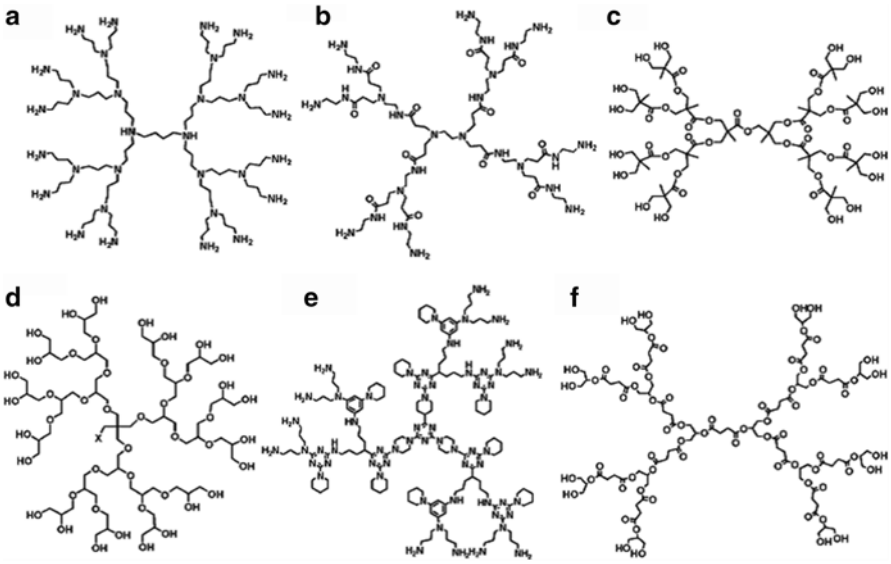

Fig. 17.1 Examples of dendritic scaffolds commonly used in drug delivery applications. (**a**) poly(propylene imine), (**b**) polyamidoamine, (**c**) polyester, (**d**) polyglycerol dendrimer, (**e**) triazine-based dendrimer, and (**f**) poly(glycerol-succinic acid) dendrimer (Adapted with permission from Ref. 3, Copyrights 2012 Royal Society of Chemistry)

17.2 Tumor Targeting

17.2.1 Enhanced Permeability and Retention (EPR) Effect and Types of Targeting

Targeting ligands can be conjugated to the multivalent dendritic surface for active targeting or the drug can be selectively delivered to the tumor tissue via the enhanced permeation and retention (EPR) effect. The EPR effect was discovered by Maeda et al. and Jain who observed that cytotoxic drugs conjugated to water soluble polymers were more effectively targeted to the tumor tissue than its free form [24]. It was investigated that most of the solid tumors possess unique characteristics such as extensive angiogenesis leading to hypervasculature, defective vascular architecture, impaired lymphatic drainage/recovery system, and greatly increased production of a number of permeability mediators which are not observed in normal tissues. All these factors ultimately lead to hyperpermeability of the tumor vasculature which allows "passive" accumulation of macromolecules in the tumor tissue and poor lymphatic drainage and resultant increased retention of macromolecules in the tumor (Fig. 17.2) [25–28]. However, for the accumulation of dendritic drug delivery systems with encapsulated or conjugated drug molecules in the tumor by EPR effect, careful attention must be paid for the construction of a monodisperse population of dendrimers in the optimal size range. For example, Haag et al. showed that

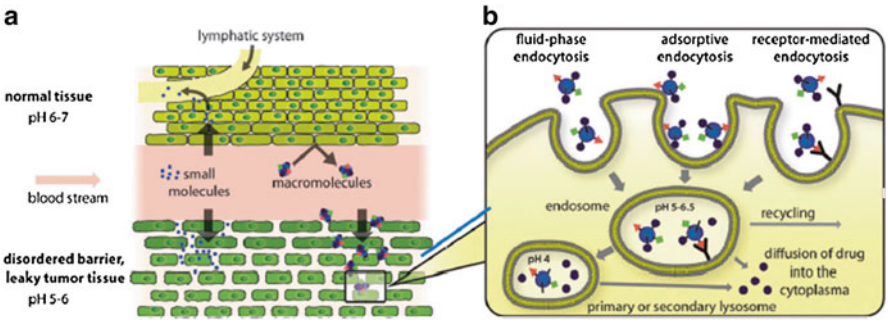

Fig. 17.2 Schematic representation of the (**a**) EPR effect and the further (**b**) cellular uptake mechanism (Adapted with permission from Ref. [3], Copyrights 2012 Royal Society of Chemistry)

dendritic polyglycerol (dPG) nanogel particles with a diameter ranging between 25 and 50 nm can be non-disruptively and efficiently taken up by cancer cells [29]. This study highlights the importance of an optimal size for the passive accumulation of a polymeric delivery system into the diseased tissues [30–33]. Secondary parameters needed to be optimized for the exploitation of the EPR effect are molecular weight, overall charge, and hydrophilic–hydrophobic characteristics of the delivery system [34, 35].

Dendritic polymers have been investigated for the tumor targeted delivery using both passive and active targeting. The EPR effect can be used for a passive targeting of drugs in tumor tissue using dendritic nanocarriers by controlling their size and physiochemical properties. Drugs can also be passively targeted by exploiting the molecular conditions of the tumor and/or organ bearing a tumor which may include but not be limited to a specific pH range and existence of certain enzymes and/or microflora in a specific organ or tumor. For example, it is well established now that the extracellular pH of solid tumors is significantly more acidic than normal tissues, with a mean pH of 6.5 in comparison to 7.4 for the blood and normal tissues [18, 36–38]. Attaching drugs to a dendritic scaffold by the low pH cleavable linker has been well explored by the Fréchet and coworkers who attached for example, the anticancer drug doxorubicin to the polyester dendritic scaffold by a low pH cleavable hydrazone linkage. The cytotoxicity of the drug was significantly reduced (80–98 %) after attachment to the polymer. However, doxorubicin could be rapidly released from the polymer in an active form at pH values found in the lysosome [39]. Furthermore, passive tumor targeting can also be done by the direct local delivery of polymeric nanocarrier conjugated anti-cancer agents directly into the tumor site [40]. The preferred tumor targeted drug delivery strategy, however, requires that the active anticancer drug should be directly taken by the target cell which is known as active targeting. The active targeting approach is based on the interactions between ligand and its cognate receptor or between specific biological pairs, e.g., antibody–antigen. Cancer cells often over express specific tumor associated antigens, carbohydrate epitopes, or growth factors receptors on their cell

surface which can be exploited for active targeting. As discussed earlier, dendritic polymers can be attached with more than one type of functionality due to their multifunctionality. This allows inclusion of ligands that can be specifically targeted to the cancer cells in addition to the encapsulated or conjugated anticancer drugs in the dendritic scaffolds. A wide range of targeting ligands have been attached to dendritic polymers and explored for active targeting which includes monoclonal antibodies (mAb), polyclonal antibodies and their fragments, carbohydrates (galactose, mannose), peptides/proteins (melanocyte stimulating hormone, transferrin, luteinizing hormone-releasing hormone (LHRH), growth factors), glycolipids, vitamins, and other ligands [27, 41, 42].

17.2.2 PAMAM Dendrimers in Tumor Targeting

PAMAM dendrimers, which are relatively non toxic, biocompatible, and a commercialized class of dendritic polymers, are under critical investigation for their practicality in cancer treatment [43, 44]. Their shelf life under ambient conditions is limited though due to the possibility of retro-Michael addition. Higher generation PAMAM dendrimers have been explored well for the targeted delivery of either encapsulated or conjugated anticancer drugs [45].

Duncan et al. for example have used a G3.5 PAMAM dendrimer for conjugation of cis-diamminedichloridoplatinum(II), which is particularly effective for testicular cancer, but its high dose side effects limit its use. This dendrimer–platinate system was water soluble and slowly released platinum in vitro. The dendrimer–Pt conjugate was also less toxic (3- to 15-fold) than cisplatin and thus showed potential for further investigation as a novel antitumor approach [46]. Recently, following the facile encapsulation approach, Wheate et al. non-covalently complexed cisplatin with half generation PAMAM dendrimers. The amount of the drug complexed was found to increase with the dendrimer generation and the dendrimer–drug complex was found to be a releasing system for cisplatin. The in vivo activity was examined using an A2780 tumor xenograft. Cisplatin, at its maximum tolerated dose of 6 mg/kg, reduced tumor size by 33 % compared to an untreated control group. The G6.5 cisplatin–dendrimer complex was administered at two doses (6 and 8 mg/kg equivalent of cisplatin). Both were well tolerated by the mice. The lower dose displayed comparable activity to cisplatin with a tumor volume reduction of 32 %, but the higher dose was significantly more active than free cisplatin with a tumor reduction of 45 % [47].

Various anticancer prodrug conjugates of PAMAM dendrimers have been successfully synthesized by conjugating anticancer drugs and targeting modalities like folic acid, antibodies, biotin, peptides (RGD, LHRH, etc.). For example, Baker et al. reported the conjugation of partially acetylated G5 PAMAM dendrimers with fluorescein isothiocyanate (FITC, an imaging agent), folic acid (FA), and paclitaxel (taxol, a chemotherapeutic drug) [48]. Folic acid is important for cell division as it participates in the biosynthesis of nucleotide bases. Folic acid receptors are membrane bound

receptors which are over expressed in a wide range of human cancers including epithelial cancer cells, such as breast, ovary, lung, kidney, head and neck, brain, and myeloid cancers [49, 50]. Recently a "one pot" approach was developed for the synthesis of targeted dendrimeric anticancer prodrug, a conjugate of G5 PAMAM dendrimer, folic acid (FA), and methotrexate (MTX), in which the ratio of FA versus MTX attached to the dendrimer can be easily tuned to achieve the desired therapeutic effect. In vitro studies performed on FA receptor-expressing KB cells show that the new conjugate has a similar affinity and cytotoxic potency to G5-FA-MTX synthesized using the traditional multiple-step approach [42]. PAMAM dendrimer itself showed some interesting effects. For example, in a very recent study, Walter et al. found that paclitaxel-conjugated G5 PAMAM dendrimers are able to affect microtubule structure via two mechanisms: (1) by stabilizing polymerizing microtubules and (2) by bundling preformed microtubules. The latter mode of action is not specific to the activity of paclitaxel, as surface neutralized G5 PAMAM dendrimers that are not conjugated with paclitaxel are also able to bundle preformed microtubules to the same degree as paclitaxel-conjugated dendrimers [51].

The Baker group has exploited fibroblast growth factor (FGF) receptor protein for the internalization of dendritic carrier into the tumor cells. FGFR is known to be over expressed in cancers such as those of the bladder, breast, prostate, and squamous cells, which can lead to tumor cell growth, tumor invasion, and metastasis [52–58]. In this study, they conjugated G5 polyamidoamine (PAMAM) dendrimer with purified recombinant fibroblast growth factor (FGF-1) and investigated the specific binding and internalization of this conjugate labeled with FITC by flow cytometry and confocal microscopic analysis in cell lines expressing FGFR. The binding and uptake of FGF-conjugated dendrimers was completely blocked by excess nonconjugated FGF-1. Confocal microscopic analysis showed cytosolic as well as nuclear localization [52].

Another important targeting ligand is biotin, which is a member of vitamin family and a growth promoter of cells. Rapidly proliferating cancerous cells require extra biotin and often overexpress biotin specific receptors on the cell surface. It has been shown that biotin-conjugated macromolecular carriers were able to increase the uptake of anticancer drugs in tumor cells [59–63]. Wen et al. for example reported the conjugation of partially acetylated G5 PAMAM dendrimers with the targeting moiety biotin and imaging moiety FITC. The bifunctional conjugate (dendrimer–biotin–FITC) exhibited much higher cellular uptake into HeLa cells than the conjugate without biotin [64].

As mentioned earlier, peptides have also been used as targeting ligands for targeting cancerous cells. Rapidly growing cancerous cells are associated with angiogenesis at a very high rate compared to normal cells. $\alpha_v\beta_3$, a transmembrane integrin receptor, is only found on the luminal surface of the endothelial cells during angiogenesis. High affinity $\alpha_v\beta_3$ selective ligands such as the Arg-Gly-Asp (RGD) peptide have been identified by phage display studies [65]. The doubly cyclized peptide (RGD-4C, containing two disulfide linkages via four cysteine residues) and a conformationally restrained RGD, binds to $\alpha_v\beta_3$ more avidly than peptides with a single disulfide bridge or linear peptides. The partially acetylated G5 PAMAM dendrimer

conjugated with Alexa Fluor 488 (AF, imaging moiety) and the doubly cyclized RGD peptide (RGD-4C). The synthesized conjugate G5-Ac-AF-RGD-4C was efficiently taken up by cells expressing $\alpha_v\beta_3$ integrin receptors as shown by flow cytometry and confocal microscopy [66].

One of the many strategies investigated for targeting cancerous cells is the use of monoclonal antibodies (mAb) that recognize and selectively bind to tumor associated antigens (TAAs) [67–70]. One such antigen for prostate cancer is the prostate-specific membrane antigen (PSMA) [71]. PSMA is a 100 kDa, type II membrane glycoprotein highly expressed by all prostate cancers as well as by nonprostatic tumor neovasculature and the vascular endothelium of virtually all solid sarcoma and carcinoma tumors [72]. Baker et al. synthesized J591 anti-PSMA (prostate specific membrane antigen) antibody dendrimer (G5 PAMAM) conjugates containing fluorophores on the dendrimer. Flow cytometry experiments have shown that the antibody–dendrimer conjugate is specifically bound by the PSMA-positive LNCaP FGC cell line but does not bind to the PC-3 cell line which does not express PSMA. This was further confirmed by confocal microscopy studies [73].

17.2.3 Dendritic Polyglycerol (dPG)–Drug Conjugates for Tumor Targeting

Dendritic polyglycerols (dPG) can be synthesized by polymerization in a controlled manner to obtain defined molecular weights with low polydispersity. Different studies have demonstrated a high in vitro and in vivo safety profile of dPGs. Haag et al. found that hyperbranched PG with molecular weight 5 kDa showed almost no toxicity at cellular level [74]. Both linear and hyperbranched PGs were reported to have similar or an even better biocompatibility profile than PEG with MW ranging from 4.2 to 670 kDa [75–78]. dPG has negligible interactions with plasma proteins. Size, surface charge, and architecture can be optimized for the cellular uptake and passive accumulation in defined tissues [3, 79, 80]. The terminal dihydroxy and linear monohydroxy groups can be functionalized or modified for the conjugation with drugs, targeting ligands, and fluorophores.

Our group also investigated the potential of dPG to target cancer cells and analyzed the correlation between the size and charge of dPGs for their intracellular delivery potential [34]. It was found that higher molecular weight dPG (MW 40–870 kDa) could passively accumulate in the cytoplasm of cancer cells after 1 h of incubation, with an increased uptake for higher molecular weight dPGs, whereas lower MW dPGs (MW: 2–20 kDa) were only detected in minimal amounts inside the cancer cells. Moreover, charged dPG derivatives, i.e., dPG-sulfate and dPG-amine showed a primarily rapid internalization inside the cytosol of cancer cells, unlike neutral dPGs with molecular weights up to 20 kDa [81].

Haag et al. have reported the use of dPGs for the delivery of anticancer drugs like doxorubicin and methotrexate using multifunctional PG–drug conjugates. A straightforward methodology was developed for the synthesis of thiolated PG by reaction

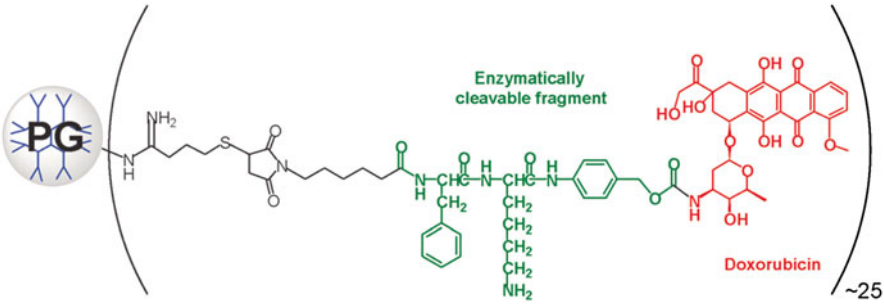

Fig. 17.3 Enzymatic cleavable prodrug derived from dendritic polyglycerol (Adapted with permission from Ref. [3]. Copyrights 2012 Royal Society of Chemistry)

of a dPG bearing 20 % amino group with iminothiolane. dPG with free thiol groups was conjugated to maleimide bearing prodrugs of doxorubicin or methotrexate which either incorporate a self-immolative para-aminobenzyloxycarbonyl (PABC) spacer coupled to the dipeptide Phe-Lys or the tripeptide D-Ala-Phe-Lys as the protease substrate. Both prodrugs were cleaved by cathepsin B, an enzyme overexpressed by several solid tumors, to release doxorubicin or a methotrexate lysine derivative (Fig. 17.3) [82]. Cytotoxicity of the conjugates towards human tumor cell lines showed that the activity of the drugs was primarily retained, which confirmed the macromolecular prodrug concept. This modular approach was found to be flexible for the coupling of different drugs, as well as targeting and solubilizing moieties [82, 83].

A similar approach was used for the conjugation of thiolated PG with the maleimide group of the 6-maleimidocaproyl (hydrazone) derivative of doxorubicin (DOXO-EMCH). The synthesized PG-Dox conjugates showed acid triggered release at pH 5.0 with only a marginal release at pH 7.4 (Fig. 17.4) [83]. Furthermore, Haag et al. reported a one-pot synthesis of polyglycerol–doxorubicin conjugate with a PEG shell (PG-Dox-PEG) using thiolated PG, DOXO-EMCH, and PEG-maleimide [84]. The synthesized PG-Dox-PEG conjugates showed optimal properties for in vitro and in vivo applications because of their high water solubility, an appropriate size for passive tumor toxicity, a high stability at physiological conditions, pronounced acid-sensitive properties, cellular internalization, and a favorable toxicity profile. Doxorubicin polyglycerol conjugates with a high drug loading ratio showed clearly improved antitumor efficacy over free doxorubicin in an ovarian xenograft tumor model (A2780), with tumor remissions upto 30 days.

Recently, Calderón et al. reported a dendritic polyglycerol-based multifunctional drug immunoconjugate that specifically targets and kills cancer cell lines expressing epidermal growth factor receptor (EGFR). The nanocarrier was provided with a dendritic core as a multifunctional anchoring point, doxorubicin (Doxo) coupled through a pH-sensitive linker, indodicarbocyanine (IDCC) as fluorescence marker, poly(ethylene glycol) as solubilizing and shielding moiety, and a scFv antibody conjugated through the SNAP-Tag technology. The study provides a proof that

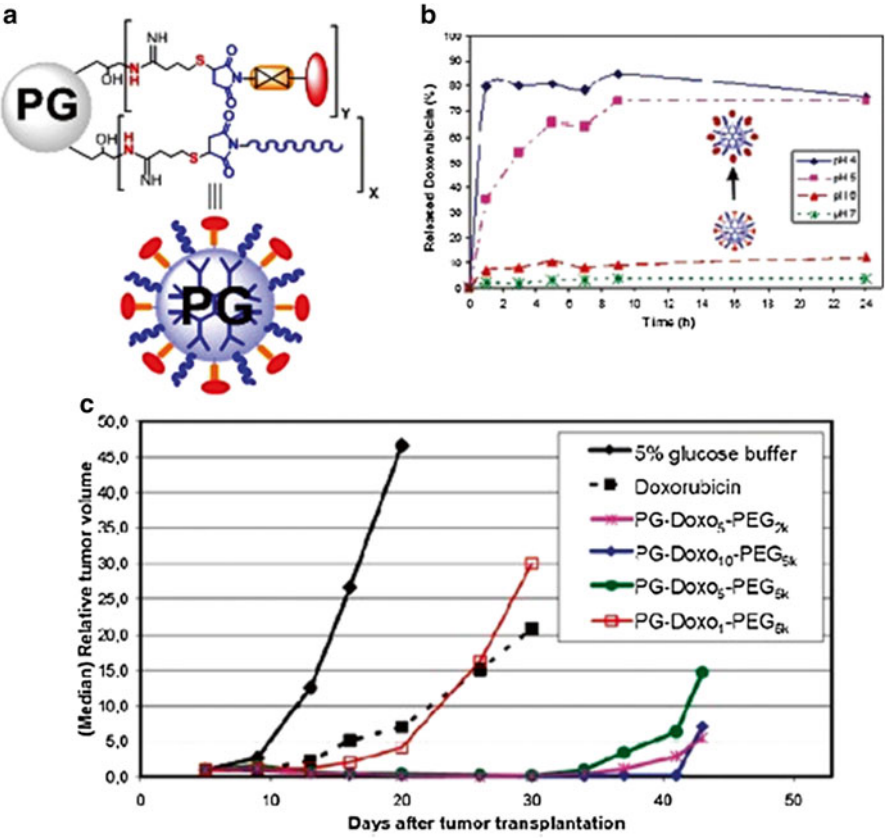

Fig. 17.4 (**a**) Schematic representation of the PG doxorubicin prodrugs. (**b**) Representative release profile of PG-Doxo5-PEG5k incubated at pH 4, 5, 6, and 7 at 37 °C. (**c**) Curves depicting tumor growth inhibition of subcutaneously growing A2780 xenografts under therapy with doxorubicin (2×8 mg/kg) and the PG doxorubicin conjugates (3×24 mg/kg doxorubicin equivalents) (Adapted with permission from Ref. [85], Copyright 2011, Elsevier)

SNAP-tag technology can be used to generate drug-carrying nanoparticles efficiently modified with single-chain antibodies to specifically target and destroy cancer cells [85]. We also have demonstrated that neutral dPGs with MW lower than 20 kDa are not passively taken up by cells [81]. Calderón et al. developed a general methodology for the synthesis of conjugates of low MW dPG (10 kDa) with homing peptides $c(RGD)_2$ and LHRH labeled with indocarbocyanine (ICC) as a fluorescent probe that enables intracellular tracking. The synthesized conjugates, c(RGD)2-dPG-ICC and LHRH-dPG-ICC, were studied for their cellular uptake in different cell lines, i.e., epithelial human lung cancer cell line A549 and human epidermoid carcinoma cells A431. LHRH-bearing dPG was taken up by both cell lines. Interesting enough, the c(RGD)2-dPG-ICC conjugate was only taken up by the cell line A549, the only one that express the αvβ3 integrin receptor which is recognized by the RGD moiety [80].

Our group reported the preparation of biodegradable dendritic polyglycerol nanogels with a disulfide linked polyglycerol (PG) structure by acid catalyzed miniemulsion polymerization with promising transport properties for cellular delivery. The size of these particles could be varied from 25 to 350 nm. These dendritic nanoparticles are stable under typical physiological conditions but efficiently break down into smaller segments under reducing intracellular conditions within reductive cell compartments, e.g., lysosomes. The less toxic and smaller fragments produced following the cleavage could be cleared by kidneys. These biodegradable polyglycerol nanogels (nPG) can be used as potential carriers for targeted delivery when functionalized with drug and targeting ligands [86].

17.2.4 Boron Neutron Capture Therapy (BNCT)

Boron neutron capture therapy (BNCT) is the selective accumulation of chemical agents containing the isotope ^{10}B in cancer cells followed by irradiation with thermal or epithermal neutrons for the treatment of cancer. This method is based on the $^{10}B(n,\alpha)7Li^{3+}$ reaction. If ^{10}B can be delivered in sufficient quantities ($>10^9$ ^{10}B atoms) to the tumor tissue, subsequent irradiation with thermal or epithermal neutrons produces highly energetic α particles and $^{7}Li^{3+}$ ions. Because of the short trajectory of these heavy particles (5–9 μm; approximately one cell diameter), radiation damage is limited to those cells containing ^{10}B. A central issue for BNCT is the precise localization of the boron containing therapeutics in the tumor [87–89]. The boronated antibodies targeted towards tumor antigens can be used to achieve high levels of boron in tumor tissues but the direct attachment of large number of boron containing molecules to antibodies can impair the solubilities and targeting efficiencies of the antibodies [20].

Multivalency and well-defined structure of dendrimers make them promising scaffolds for use as boron carriers which can be conjugated with antibodies for tumor targeted boron delivery. PAMAM dendrimers have been used as immunoconjugates for delivering high concentrations of boron in tumors. For example, Darby et al. conjugated isocyanato polyhydral borane [$Na(CH_3)_3NB_{10}H_8NCO$] with G2 and G4 PAMAM dendrimers followed by conjugation with monoclonal antibody 1B16-6 for selective targeting to the murine B16 melanoma. The immunoconjugate retained a high level of immunoreactivity and had a strong propensity to localize in the liver and spleen [90]. Boronated PAMAM dendrimers conjugated with EGF were found to target epidermal growth factor (EGF) receptors, resulting in accumulation of boron in cell lysosomes in vitro. In vivo studies showed, however, that these boron carriers were taken up by the liver resulting in low accumulation of boron in the tumor [91, 92]. On the other hand, G5 PAMAM dendrimers carrying 1100 boron atoms to cetuximab, a monoclonal antibody specific to the EGF receptor, showed a tenfold greater accumulation of boron in brain tumor than in normal brain tissue after intratumoral injection [93]. Polylysine dendrimers with several terminal carborane moieties and linked to antibody fragments also revealed promising perspectives for applications in BNCT [94].

17.3 Targeted Gene Delivery

Gene delivery is the process of introducing foreign genetic materials (DNA, RNA, or antisense oligonucleotides (AODNs)) as medicines into the host cells to cure disease. Positively charged dendritic polymers have been used as nonviral vectors for the targeted delivery of genetic material into the diseased cells [95]. Electrostatic interactions between negatively charged phosphate backbones of DNA/RNA strand with dendritic polymers carrying positively charged surface groups for example, dendritic polyamines can result in a polyplex formation. The polyplex (nuclear material–dendrimer complex) is endosomally taken up by the target cells, and following lysosomal fusion and subsequent endosomal escape, the genetic materials are released from the complex in the vicinity of the nucleus (Fig. 17.5) [31]. The "Proton-sponge" effect of dendritic polyamines plays an important role in release of genetic material into the cell cytoplasm. For example, PAMAM dendrimers possess secondary and tertiary amines in their core in addition to primary amines at the surface. This means dendrimers act like a buffer system inside endosomes or endolysosomes (which contain digestive enzymes), leading to the pumping of protons and concomitant influx of chloride ions. The ionic strength inside these organelles will

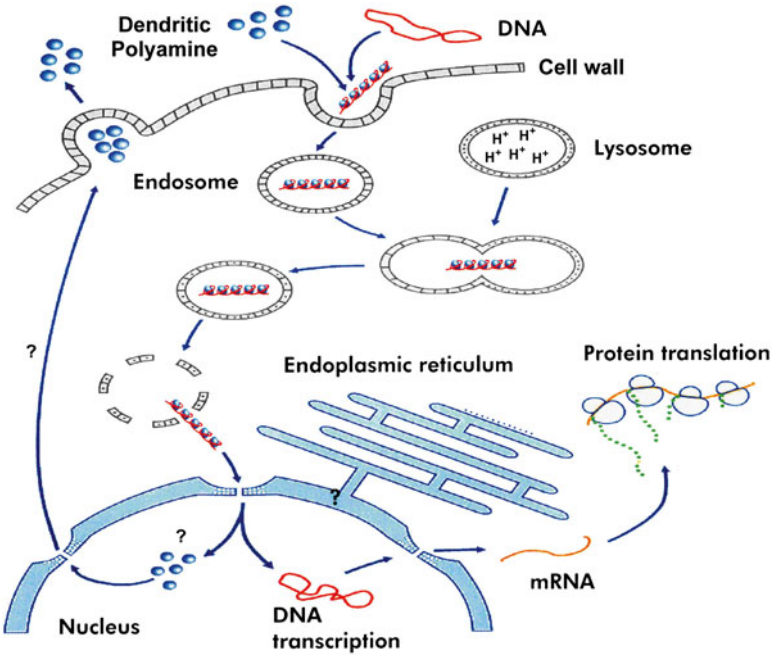

Fig. 17.5 Intracellular uptake of therapeutic DNA or RNA complexed with polycationic polymers, i.e., dendritic polyamines (Adapted with permission from Ref. [31], Copyright 2006 John Wiley & Sons, Inc)

then increase and eventually cause their osmotic rupture and the release of nuclear material or dendrimer/nuclear material complex into the cytoplasm [96, 97].

Although the therapeutic benefit of dendritic polyamines as nonviral gene transfer vectors has been demonstrated in animal models, the gene delivery efficiency of the nonviral approaches remains a key obstacle for clinical applications [98]. It was found that the proton sponge effect could be increased by oligoethyleneimine (OEI) or poly(propylene imine) (PPI) dendrimer modification leading to high transfection efficiency in vitro [99, 100]. Haag et al. reported that dendritic polyglycerols with oligoamine shells prepared by linking pentaethylenehexamine (PEHA) with dendritic polyglycerol (dPG) via biodegradable carbamate linkage showed low toxicity and high siRNA transfection efficiency when tested using human epithelial carcinoma cells (HeLaS3) with different proteins (Lamin, CDC2, MAPK2) [101].

Furthermore, dendritic polymers can be conjugated with respective targeting ligands for the active accumulation of genetic material into the targeted cells. PAMAM dendrimer–RGD conjugates complexed with siRNA, for example, enhanced the siRNA delivery and reduced the progression of angiogenesis in the treatment of solid tumors [102–104]. Zhang et al. evaluated the efficiency of folate–polyamidoamine dendrimer conjugates (FA-PAMAM) for the in situ delivery of therapeutic antisense oligonucleotides (ASODN) to inhibit the growth of C6 glioma cells. ASODN from the FA-PAMAM knocked down EGFR expression in C6 glioma cells, both in vitro and in vivo [105].

It was observed that PEGylating of the dendrimer surface shields the positive charge of the dendrimer thus reducing nonspecific interactions with cell membranes and attenuating oxidative stress on the cell [106, 107]. Pegylation also increases the circulation time of the dendrimer in blood by avoiding the interaction of dendrimer surface amino groups with serum proteins [108, 109]. A prolonged circulation time in blood enhances the drug accumulation in diseased tissues (tumor or inflammatory site) via the EPR effect [110, 111]. PEGylated dendrimer–RGD conjugates are a well-designed delivery adjunct that improves target selectivity as well as stability of the dendrimer complex with nucleic acids. Efficient intramuscular gene expression was mediated by PEG-conjugated PAMAM dendrimer [112, 113].

With targeting ligands attached, the nanospace of the dendrimer interior can be used as a vehicle for encapsulation of chemotherapeutic agents (e.g., doxorubicin or paclitaxel) while the outer shell just beneath the dendrimer surface serves a nucleic acid shielding from the action of endo/exo nucleases [112]. In a recent study a G3 poly(L-lysine) dendrimer with a silsesquioxane cubic core (nanoglobules) was conjugated with PEG and RGD for codelivery of doxorubicin (DOX) and siRNA. Doxorubicin (DOX) was coupled to the RGD targeted nanoparticle via a degradable disulfide spacer to give G3-[PEG-RGD]-[DOX]; G3-[PEG-RGD]-[DOX] was further complexed with siRNA [103]. The siRNA complexes of the targeted conjugate resulted in significantly higher gene silencing efficiency in U87-Luc cells than those of control conjugates G3-[PEG-RGD] and G3-[DOX]. These dendritic nanocarriers are promising for the codelivery of nucleic acids and chemotherapeutic agents.

Our group reported the synthesis of a single dendritic polyglycerol amine (dPG-NH2) which showed promising properties as a prospective system for gene delivery,

namely, because of its high charge with a relative low cytotoxicity and an optimal charge/pH behavior so far as the buffering capacity is concerned [34]. Dendritic polyglycerolamine (dPG-NH_2) was able to complex siRNA thus yielding slightly positively charged globular polyplexes which exhibited a knockdown efficiency comparable to that of the HiPer-Fect for the proteins, Lamin, CDC2, and MAPK2 in HeLAS3 cells. In a comparison of silencing efficiency and cytotoxicity with poly(ethylene imine) (PEI) derivatives, the polyglycerolamine architecture showed a better toxicity profile at concentrations relevant for its activity. It was found that the siRNA polyplex was internalized into glioblastoma cells within 24 h by the endosome–lysosome mediated system. The siRNA-PG-NH_2 polyplex resulted in major silencing and no apparent toxicity when administered intratumorally to a tumor bearing mice. Polyglycerolamine carrier delivered siRNA passively into the tumor tissues by the EPR effect, as high levels of fluorescently labelled siRNA were detected in the tumor but not in the other healthy organs examined [114].

Recently, dPG-NH2 was successfully used in xenografted nude mice to deliver siRNA that down-regulate the mRNA expression of ferrochelatase (FECH). The fluorescence imaging results on animals with xenografted tumors demonstrated that dPG-NH_2 improved the local bioavailability of siRNA within the tumor tissue and facilitated the transfer of siRNA across the cell membrane. Moreover, siRNA transfected in this way reached the cytoplasm and was effective in silencing its target FECH as proven by the high time dependent fluorescence emission of protoporphyrin IX (PpIX), a fluorescent metabolite of the heme synthesis [115].

17.4 Brain Targeted Drug Delivery

Delivery of drugs or genes to brain for treatment of brain diseases is one of the biggest challenges in targeted drug delivery. It is because of the blood–brain barrier (BBB), blood–cerebrospinal fluid barrier (BCSFB), and efflux mechanism (P-glycoprotein; P-gp) [116]. The blood–brain barrier (BBB) is a physical and biological barrier consisting of an endothelial cell monolayer combined with tight intracellular junctions, pericytes, and astrocytes [117]. It prevents the introduction of harmful blood-borne substances and restricts the movement of ions and fluid into the brain to ensure an optimal environment for brain function [118]. The surface area of the BBB has been estimated to be 5,000-fold greater than that of the BCSFB, and therefore the BBB is considered to be the main route for the uptake of endogenous and exogenous ligands into the brain parenchyma [119–122]. Efflux transport systems at the BBB and BCSFB provide a protective barrier function by removing drugs from the brain or cerebrospinal fluid and transferring them to the systemic circulation [123]. In the human brain, the total length of capillaries is about 400 miles, so a drug or gene will instantaneously distribute throughout the CNS once the BBB is traversed. But more than 98 % of candidate drugs including gene drugs have been halted mid-development due to the poor permeability of the BBB, presenting a major problem to the pharmaceutical industry [124, 125].

Both viral and non-viral vectors have been used for the brain targeted delivery of genes as drugs, and most of them are internalized into cells by an endocytic mechanism [126–131]. Viral vectors are highly immunogenic in humans and invoke severe inflammatory responses in the brain leading to demyelination. Therefore, the goal of brain targeted drug delivery should be a non-viral, non-invasive approach through exploitation of the various influx transport systems depicted within the cerebral endothelial, including carrier-mediated transports, receptor-mediated endocytosis, and adsorptive-mediated endocytosis. Non-viral vectors were found to be much safer but their transfer efficiency is comparatively lower. Nowadays, nano-sized carriers, especially polymeric carriers (liposomes, nanoparticles, and dendrimers), are being used widely and are under investigation with or without coating or coupling with ligands to offer a sustained level of drug and to accomplish cellular target with improved specificity [132–135]. It has been reported that different receptors are present on the luminal endothelial plasma membranes, including the transferrin receptor, the insulin receptor, and the low-density lipoprotein receptor-related protein [136, 137]. Dendrimers can be anchored with ligands that bind exclusively to the receptors over expressed on BBB and therefore may elicit receptor-mediated endocytosis. Apolipoproteins, e.g., ApoE are believed to be adsorbed on the surfactant polysorbate 80 (P80) anchored nanocarriers, which could be responsible for the interaction with low density lipoproteins (LDL) receptors on BBB and subsequent endocytosis [138]. Jain et al. reported the synthesis of P80 anchored PPI dendritic nanoconjugate for targeting anticancer drug, docetaxel (DTX) to the brain tumor in vitro (cytotoxicity study U87MG human glioblastoma cell line) and in vivo. The in vivo anti cancer activity in brain tumor bearing rats revealed that DTX loaded P80 conjugated dendrimers reduced the tumor volume significantly (>50 %). The median survival time for brain tumor bearing rats treated with DTX-P80-PPI dendrimers (42 days) was extended substantially compared to DTX-PPI (23 days), receptor blocked group (15 days), and free DTX (18 days). Gamma scintigraphy and biodistribution studies further confirmed the targeting efficiency and higher biodistribution of ligand conjugated dendrimer into the brain [139].

Another example is the dual targeting carrier system reported by Wei et al. based on G4 PAMAM dendrimers with Transferrin (Tf) and Tamoxifen (TAM) as the targeting groups for strengthening the BBB transporting ability and concentrating the drugs in the glioma cells. As an endogenous cellular transport ligand, Tf has been widely studied in the brain-targeted drug delivery systems [140] because Tf receptor is over expressed on the brain capillary endothelium and many malignant cells [141–143]. Tamoxifen (TAM), known as an estrogen receptor modulator, shows the ability to inhibit multiple drug resistance (MDR) and improve the BBB transporting [144, 145]. The dual-targeting carrier was synthesized by peripherally linking DOXs through an acid labile acyl hydrazone bond in order to control the drug release, conjugating Tf and PEG chains for enhancing the ability of crossing the BBB, internalizing in glioma cells and improving the biocompatibility. Meanwhile, TAMs were embedded in the interior of the dendrimers as the MDR inhibitors. The carrier was internalized into C6 glioma cells upon crossing the BBB model by the coactions of Tf receptor-mediated endocytosis and the inhibition effect of TAM to the drug efflux transports.

Moreover, there was an in vitro accumulation of DOX in the avascular C6 glioma spheroids that effectively reduced tumor volume [146]. In addition to dendrimers with targeting ligands, fluorescently labeled G4 PAMAM dendrimers (FITC-PAMAM) were found to be effective in targeting neuroinflammation in a rabbit model of cerebral palsy following subarachnoid administration. The dendrimers with no targeting ligands were localized in activated microglia and astrocytes (cells responsible for neuroinflammation), even in regions far from the site of injection, in newborn rabbits with maternal inflammation-induced cerebral palsy. This intrinsic ability of dendrimers to localize inactivated microglia and astrocytes can enable a targeted delivery of therapeutics in disorders such as cerebral palsy, Alzheimer's, and multiple sclerosis [147].

PAMAM dendrimers modified with peptide targeting ligands, have been explored as non-viral vectors for delivering genes to the brain by receptor-mediated transcytosis systems existing at the BBB [148, 149]. Angiopep is a high brain penetration peptide, which targets to the low-density lipoprotein receptor-related protein-1. Angiopep-conjugated, PEG-modified PAMAM (PAMAM-PEG-Angiopep) was found to be efficient for brain-targeting gene delivery both in vitro and in vivo. PAMAM-PEG-Angiopep/DNA NPs were internalized by brain capillary endothelial cells (BCECs) through clathrin- and caveolae-mediated energy depending endocytosis, also partly through macropinocytosis. The angiopep-modified NPs showed higher efficiency in crossing the BBB than unmodified NPs in an in vitro model, and accumulated in vivo more in the brain. The angiopep-modified NPs also showed higher efficiency in gene expression in the brain than the unmodified NPs [150]. Lactoferrin, a newly explored brain-targeting ligand, was coupled to the dendrimer for targeted delivery of DNAs and the lactoferrin-coupled dendrimer was reported to have high BBB-crossing efficiency. The lactoferrin (Lf) and transferrin (Tf) conjugated PAMAM dendrimers via PEG spacer were synthesized and compared for their brain uptake and transfection efficiencies. The brain uptake of PAMAM-PEG-Lf was 2.2-fold compared to that of PAMAM-PEG-transferrin (Tf) in vivo. The transfection efficiency of PAMAM-PEG-Lf/DNA complex was higher than that of PAMAM-PEG-Tf/DNA complex in vitro and in vivo [151].

Sarin and coworkers functionalized PAMAM dendrimers generations 1 through 8 with gadolinium and rhodamine B for both magnetic resonance and fluorescence imaging. They found that following intravenous injection, the functionalized dendrimers with a diameter of less than approximately 11.7–11.9 nm were able to traverse the pores of the blood–brain tumor barrier of RG-2 malignant gliomas [152]. This finding could serve as a guide for future nanoparticle-based chemotherapy design.

17.5 Drug Delivery Through the Skin

Topical treatment of skin diseases always appears favorable due to the lower risk of systemic side effects. The outermost layer of skin, the stratum corneum (SC), is essential for a normal barrier function of the skin, and skin lipids also greatly

contribute to the penetration barrier. Various lipid nanoparticles, which may allow lipid exchange with skin surfaces, have been found to be promising. These include liposomes [153, 154], solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), lipid microspheres [155, 156], microemulsions [157], and hexagonal phase nanodispersions [158], which attach themselves to the skin surface. Indeed, the nature of the lipid particle and the mode of drug interaction with the lipid matrix have proven to be quite relevant [159].

Besides lipid nanoparticles, dendritic nanoparticles have also become popular in skin drug delivery mainly due to their small size and highly functional surfaces [21, 160]. Skin delivery enhancement by dendritic polymers depends on drug–dendrimer–skin interactions. Dendrimers may act as a drug release modifier and speed up the drug dissolution that is the rate-limiting step of percutaneous drug absorption. Certain low generation dendrimers may impair the stratum corneum barrier function, in the presence of potent vehicles. Moreover, properties of the dendrimers can be tailored to preferably penetrate the skin via the follicular route [161]. Regardless of the diversity of dendrimers, only two types of dendrimers (PAMAM and PEGylated PG) have been investigated to modulate drug delivery to the skin.

Recently, Hong et al. reported that smaller G2 PAMAM dendrimers penetrate the skin layers more efficiently than the bigger G4 dendrimers. It was found that G2 PAMAM dendrimers, surface-modified by either acetylation or carboxylation, exhibit increased skin permeation and tend to diffuse through an extracellular pathway. In contrast, amine terminated dendrimers show enhanced cell internalization and skin retention but a reduced skin permeation [162]. All these investigations provide a design guideline for engineering PAMAM dendrimers as a potential transdermal delivery vector. PAMAM dendrimers have been proven to effectively improve transdermal penetration of 5-fluorouracyl, ketoprofen, diflunisal, and 5-ALA [163]. More recently, Radej et al. assessed the ability of G3 and G4 PAMAM dendrimers to facilitate transdermal delivery of 8-methoxypsoralen (8-MOP) in vivo, which revealed an enhanced permeation of the 8-methoxypsoralen (8-MOP) to the deeper layers of the skin and significantly higher concentration in comparison with standard 8-MOP solution [164].

Our group reported the synthesis of dendritic core-multishell (CMS) nanotransporters using hyperbranched polymeric cores composed of polyglycerol surrounded by a double-layered shell consisting of a C18-alkyl chain and of monomethoxy poly(ethylene glycol) [165, 166]. These CMS nanotransporters (20–30 nm) were compared with solid lipid nanoparticles (SLNs, 150–170 nm) for their skin transport properties using a model dye Nile Red. CMS nanotransporters were found to be much more effective than SLN for the skin penetration. Using CMS, dye concentration increased eightfold in the stratum corneum and 13-fold in the epidermis compared to a regular cream. Despite SLN degradation at the stratum corneum surface, SLN enhanced skin penetration less efficiently (3.8- and 6.3-fold) [167]. These CMS nanotransporters were also explored for the delivery of more hydrophilic agents into the skin using the dye rhodamine B as model compound, and found to be more efficient than the SLNs (250–340 nm) [168].

17.6 Targeting Inflammation

Several investigations proved the potential of dendritic polymers in the targeted delivery of anti-inflammatory drugs and also controlling pro-inflammatory responses. Anionic dendrimers with or without targeting ligands were found to accumulate in the inflammatory tissues inducing anti-inflammatory responses. Selected examples will be discussed here.

Hydroxyl terminated PAMAM dendrimer conjugated with flocinolone acetonide was found to be effective in targeted intravitreal therapy for sustained attenuation of neuroinflammation in retinal degeneration. The conjugate released drug in a sustained manner over a period of 90 days. Kannan et al. showed that upon intravitreal administration, PAMAM dendrimers selectively localize within activated outer retinal microglia in two rat models of retinal degeneration, but not in the retina of healthy controls. The in vivo studies suggest that PAMAM dendrimers (with no targeting ligands) have an intrinsic ability to selectively localize in activated microglia, and can deliver drugs inside these cells for a sustained period for the treatment of retinal neuroinflammation [169].

Diwan et al. investigated folate-PEG-(G3.5) PAMAM conjugates for the inflammatory tissue specific drug delivery of indomethacin in the arthritis rats. Among the different folate-PEG-PAMAM conjugates, the conjugate with 7 folate-PEG molecules exhibited better overall drug targeting efficiency (3.44) and higher relative exposure of drug to the inflammatory region (2.37). This folate-PEG-PAMAM conjugate remains the ideal candidate for site-specific drug delivery with high efficiency to target the inflammatory tissues accompanied by reduced side-effects [170]. Hayder et al. illuminated the nano-therapeutic potential of phosphorus containing dendrimers, in particular dendrimers with anionic azabisphoshonate endings, for the modulation of innate immunity through their effect on monocytes. Dendrimer ABP (dendrimers with anionic Azabisphosphonate endings) inhibited osteoclastogenesis and the secretion of proinflammatory cytokines, both of which are underlying monocyte-dependent processes in rheumatoid arthritis inflammation and bone damage [171, 172]. G3.5 PAMAM dendrimers conjugated with D(+)-glucosamine inhibited Toll-like receptor 4-mediated lipopolysaccharide induced synthesis of pro-inflammatory chemokines (MIP-1α, MIP-1β, IL-8) and cytokines (TNF-α, IL-1β, IL-6) from human dendritic cells and macrophages but allowed upregulation of the costimulatory molecules CD25, CD80, CD83, and CD86 [173].

Dendritic polymers have been proven to be potential candidates for the treatment of rheumatoid arthritis (RA). Baker et al. reported folic acid and methotrexate (MTX) conjugated G5 PAMAM dendrimer (FA-PAMAM-MTX) as a potent anti-inflammatory agent. FA-PAMAM-MTX bound and internalized in a receptor-specific manner into both folate receptor β-expressing macrophage cell lines and primary mouse macrophages, and beneficially suppressed inflammatory changes associated with type II collagen-induced arthritis in rats [174]. Multivalent polyglycerol sulfate (dPGS) has been shown to be a multivalent inhibitor of inflammation [175]. Recently, our group investigated the potential of anionic multifunctional

dendritic polyglycerol sulfate (dPGS) for the selective targeting of inflammation in an animal model of rheumatoid arthritis (RA) using near-IR fluorescence imaging. A study using fluorescently labeled dPGS demonstrated a fast and selective uptake into the diseased joints. The sulfated dendritic polglycerol deposited in the inflammatory infiltrate in the synovial membrane, whereas nonsulfated control was not detected in association with disease [176]. Hence, this type of multivalent polyanion is an alternative to current bioconjugates and provides future options for targeted imaging and drug delivery [177].

References

1. Menjoge AR, Kannan RM, Tomalia DA (2010) Dendrimer-based drug and imaging conjugates: design considerations for nanomedical applications. Drug Discov Today 15:171–185
2. Lee CC, MacKay JA, Fréchet JMJ, Szoka FC (2005) Designing dendrimers for biological applications. Nat Biotechnol 23:1517–1526
3. Khandare J, Calderón M, Dagia NM, Haag R (2012) Multifunctional dendritic polymers in nanomedicine: opportunities and challenges. Chem Soc Rev 41:2824–2848
4. Gingras M, Raimundo JM, Chabre YM (2007) Cleavable dendrimers. Angew Chem Int Ed 46:1010–1017
5. Jansen JFGA, de Brabander-van den Berg EMM, Meijer EW (1994) Encapsulation of guest molecules into a dendritic box. Science 266:1226–1229
6. Gillies ER, Fréchet JMJ (2002) Designing macromolecules for therapeutic applications: polyester dendrimers-poly(ethylene oxide) "Bow-Tie" hybrids with tunable molecular weight and architecture. J Am Chem Soc 124:14137–14146
7. Gillies ER, Dy E, Fréchet JMJ, Szoka FC (2005) Evaluation of polyester dendrimer: poly(ethylene oxide) "BowTie" hybrids with tunable molecular weight and architecture. Mol Pharm 2:129–138
8. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, Roeck J, Ryder J, Smith P (1985) A new class of polymers: starburst-dendritic macromolecules. Polym J 17:117–132
9. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, Roeck J, Ryder J, Smith P (1986) Dendritic macromolecules: synthesis of starburst dendrimers. Macromolecules 19: 2466–2468
10. Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W, Duncan R (2000) Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. J Control Release 65:133–148
11. Ziemba B, Janaszewska A, Ciepluch K, Krotewicz M, Fogel WA, Appelhans D, Voit B, Bryszewska M, Klajnert B (2011) In vivo toxicity of poly(propyleneimine) dendrimers. J Biomed Mater Res A 99:261–268
12. Rao C, Tam JP (1994) Synthesis of peptide dendrimer. J Am Chem Soc 116:6975–6976
13. Hawker CJ, Fréchet JMJ (1990) Preparation of polymers with controlled molecular architecture. A new convergent approach to dendritic macromolecules. J Am Chem Soc 112:7638–7647
14. Grinstaff MW (2002) Biodendrimers: new polymeric biomaterials for tissue engineering. Chemistry 8:2839–2846
15. Boysen MK, Elsner K, Sperling O, Lindhorst TK (2003) Glycerol and glycerol glycol glycodendrimers. Eur J Org Chem 22:4376–4388
16. Posocco P, Pricl S, Jones S, Barnard A, Smith DK (2010) Less is more - multiscale modelling of self-assembling multivalency and its impact on DNA binding and gene delivery. Chem Sci 1:393–404

17. Wilms D, Stiriba SE, Frey H (2010) Hyperbranched polyglycerols: from the controlled synthesis of biocompatible polyether polyols to multipurpose applications. Acc Chem Res 43:129–141
18. Calderón M, Quadir MA, Strumia M, Haag R (2010) Functional dendritic polymer architectures as stimuli-responsive nanocarriers. Biochimie 92:1242–1251
19. Simanek EE, Abdou H, Lalwani S, Lim J, Mintzer M, Venditto VJ, Vittur B (2010) The 8 year thicket of triazine dendrimers: strategies, targets and applications. Proc R Soc A 466: 1445–1468
20. Gillies ER, Fréchet JMJ (2005) Dendrimers and dendritic polymers in drug delivery. Drug Discov Today 10:35–43
21. Esfand R, Tomalia DA (2001) Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. Drug Discov Today 6:427–436
22. Haag R, Sunder A, Stumbé JF (2000) An approach to glycerol dendrimers and pseudo-dendritic polyglycerols. J Am Chem Soc 122:2954–2955
23. Carnahan MA, Grinstaff MW (2001) Synthesis and characterization of polyether-ester dendrimers from glycerol and lactic Acid. J Am Chem Soc 123:2905–2906
24. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46:6387–6392
25. Grecol F, Vicent MJ (2008) Polymer-drug conjugates: current status and future trends. Front Biosci 13:2744–2756
26. Iyer AK, Khaled G, Fang J, Maeda H (2006) Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discov Today 11:812–818
27. Torchilin V (2011) Tumor delivery of macromolecular drugs based on the EPR effect. Adv Drug Deliv Rev 63:131–135
28. Fang J, Nakamura H, Maeda H (2011) The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Adv Drug Deliv Rev 63:136–151
29. Sisson AL, Steinhilber D, Rossow T, Welker P, Licha K, Haag R (2009) Biocompatible functionalized polyglycerol microgels with cell penetrating properties. Angew Chem Int Ed 48:7540–7545
30. Haag R, Kratz F (2006) Polymere therapeutika: konzepte und anwendungen. Angew Chem 118:1218–1237
31. Haag R, Kratz F (2006) Polymer therapeutics: concepts and applications. Angew Chem Int Ed 45:1198–1215
32. Maeda H, Greish K, Fang J (2006) The EPR effect and polymeric drugs: a paradigm shift for cancer chemotherapy in the 21st century. Adv Polym Sci 193:103–121
33. Peer D, Karp JM, Hong S, Faro Khzad OC, Margalit R, Langer R (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2:751–760
34. Khandare J, Mohr A, Calderón M, Welker P, Licha K, Haag R (2010) Structure- biocompatibility relationship of dendritic polyglycerol derivatives. Biomaterials 31:4268–4277
35. Khandare J, Minko T (2006) Polymer-drug conjugates: progress in polymeric prodrugs. Prog Polym Sci 31:359–397
36. Engin K, Leeper DB, Cater JR, Thistlethwaite AJ, Tupchong L, McFarlane JD (1995) Extra cellular pH distribution in human tumours. Int J Hyperthermia 11:211–216
37. van Sluis R, Bhujwalla ZM, Ballerteros P, Alverez J, Cerdan S, Galons JP, Gillies RJ (1999) In vivo imaging of extracellular pH using 1H MSRI. Magn Reson Med 41:743–750
38. Ojugo ASE, Mesheehy PMJ, McIntyre DJO, McCoy C, Stubbs M, Leach MO, Judson IR, Griffiths JR (1999) Measurement of the extracellular pH of solid tumors in mice by magnetic resonance spectroscopy: a comparison of exogenous 19F and 31P probes. NMR Biomed 12:495–504
39. Padilla De Jesús OL, Ihre HR, Gagne L, Fréchet JMJ, Szoka FC Jr (2002) Polyester dendritic systems for drug delivery applications: in vitro and in vivo evaluation. Bioconjug Chem 13: 453–461

40. Minko T, Khandare J, Jayant S (2007). In: Macromolecular Engineering: From Precise Macromolecular Synthesis to Macroscopic Material Properties and Application. Matyjaszewski K, Gnanou Y, Leibler L, editors. vol. 4th. Wiley-VCH Verlag GmbH & Co.; Weinheim: pp. 2541–2595
41. Minko T (2004) Drug targeting to the colon with lectins and neoglycoconjugates. Adv Drug Deliv Rev 56:491–509
42. Zhang Y, Thomas TP, Desai A, Zong H, Leroueil PR, Majoros IJ, Baker JR Jr (2010) Targeted dendrimeric anticancer prodrug: a methotrexate-folic acid-poly(amidoamine) conjugate and a novel, rapid, "One Pot" synthetic approach. Bioconjug Chem 21:489–495
43. Patri AK, Majoros IJ, Baker JR Jr (2002) Dendritic polymer macromolecular carriers for drug delivery. Curr Opin Chem Biol 6:466–471
44. Roberts JC, Adams YE, Tomalia D, Mercer-Smith JA, Lavallee DK (1990) Using starburst dendrimers as linker molecules to radiolabel antibodies. Bioconjug Chem 1:305–308
45. Patri AK, Kukowska-Latallo JF, Baker JR Jr (2005) Targeted drug delivery with dendrimers: comparison of the release kinetics of covalently conjugated drug and non-covalent drug inclusion complex. Adv Drug Deliv Rev 57:2203–2214
46. Malik N, Evagorou EG, Duncan R (1999) Dendrimer-platinate: a novel approach to cancer chemotherapy. Anticancer Drugs 10:767–776
47. Kirkpatrick GJ, Plumb JA, Sutcliffe OB, Flint DJ, Wheate NJ (2011) Evaluation of anionic half generation 3.5–6.5 poly(amidoamine) dendrimers as delivery vehicles for the active component of the anticancer drug cisplatin. J Inorg Biochem 105:1115–1122
48. Majoros IJ, Myc A, Thomas T, Mehta CB, James R, Baker JR Jr (2006) PAMAM dendrimer-based multifunctional conjugate for cancer therapy: synthesis, characterization, and functionality. Biomacromolecules 7:572–579
49. Ross JF, Chaudhuri PK, Ratnam M (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. Cancer 73:2432–2443
50. Campbell IG, Jones TA, Foulkes WD, Trowsdale J (1991) Folate-binding protein is a marker for ovarian cancer. Cancer Res 5:5329–5338
51. Cline EN, Li M, Choi SK, Herbstman JF, Kaul N, Meyhöfer E, Skiniotis G, Baker JR, Larson RG, Walter NG (2013) Paclitaxel-onjugated PAMAM dendrimers adversely affect microtubule structure through two independent modes of action. Biomacromolecules 14:654–664
52. Mason I (2003) Fibroblast growth factors. Curr Biol 13:R346
53. Thomas TP, Shukla R, Kotlyar A, Kukowska-Latallo J, Baker JR Jr (2010) Dendrimer-based tumor cell targeting of fibroblast growth factor-1. Bioorg Med Chem Lett 20:700–703
54. Ornitz DM, Itoh N (2001) Fibroblast growth factors. Genome Biol 2:3005.1–3005.12
55. Dickson C, Spencer-Dene B, Dillon C, Fantl V (2000) Tyrosine kinase signalling in breast cancer Fibroblast growth factors and their receptor. Breast Cancer Res 2:191–196
56. Dailey L, Ambrosetti D, Mansukhani A, Basilico C (2005) Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev 16:233–247
57. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M (2005) Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev 16:159–178
58. Jeffers M, LaRochelle WJ, Lichenstein HS (2002) Fibroblast growth factors in cancer: therapeutic possibilities. Expert Opin Ther Targets 6:469–482
59. Russell-Jones G, McTavish K, McEwan J (2004) Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumors. J Inorg Biochem 98:1625–1633
60. Marek M, Kaiser K, Gruber HJ (1997) Biotinepyrene conjugates with poly(ethylene glycol) spacers are convenient fluorescent probes for avidin and streptavidin. Bioconjug Chem 8:560–566
61. Cannizzaro SM (1998) A novel biotinylated degradable polymer for cell interactive applications. Biotechnol Bioeng 58:529–535
62. Na K, Lee TB, Park K-H, Shin E-K, Lee Y-B, Cho H-K (2003) Self-assembled nanoparticles of hydrophobically-modified polysaccharide bearing vitamin H as a targeted anti-cancer drug delivery system. Eur J Pharm Sci 18:165–173

63. Mishra PR, Jain NK (2002) Biotinylated methotrexate loaded erythrocytes for enhanced liver uptake. 'a study on the rat'. Int J Pharm 231:145–153
64. Yang W, Cheng Y, Xu T, Wang X, Wen L-P (2009) Targeting cancer cells with biotin-dendrimer conjugates. Eur J Med Chem 44:862–868
65. Pasqualini R, Koivunen E, Ruoslahti E (1997) αv Integrins as receptors for tumor targeting by circulating ligands. Nat Biotechnol 15:542–546
66. Shukla R, Thomas TP, Jennifer Peters J, Alina Kotlyar A, Andrzej Myc A, Baker JR Jr (2005) Tumor angiogenic vasculature targeting with PAMAM dendrimer–RGD conjugates. Chem Commun 46:5739–5741
67. Laheru D, Jaffee EM (2005) Immunotherapy for pancreatic cancer-science driving clinical progress. Nat Rev Cancer 5:549–567
68. Harris M (2004) Monoclonal antibodies as therapeutic agents for cancer. Lancet Oncol 5:292–302
69. Lin MZ, Teitell MA, Schiller GJ (2005) The evolution of antibodies into versatile tumor-targeting agents. Clin Cancer Res 11:129–138
70. Zhang J-Y (2004) Tumor-associated antigen arrays to enhance antibody detection for cancer diagnosis. Cancer Detect Prev 28:114–118
71. Chang SS, Gaudin PB, Reuter VE, Heston WDW (2000) Prostate-specific membrane antigen: present and future application. Urology 55:622–629
72. Chang SS, O'Keefe DS, Bacich DJ, Reuter VE, Heston WD, Gaudin PB (1999) Prostate-specific membrane antigen is produced in tumor-associated neovasculature. Clin Cancer Res 5:2674–2681
73. Patri AK, Myc A, Beals J, Thomas TP, Bander NH, Baker JR Jr (2004) Synthesis and in vitro testing of J591 antibody-dendrimer conjugates for targeted prostate cancer therapy. Bioconjug Chem 15:1174–1181
74. Frey H, Haag R (2002) Dendritic polyglycerol: a new versatile biocompatible material. Rev Mol Biotechnol 90:257–267
75. Kainthan RK, Brooks DE (2007) In vivo biological evaluation of high molecular weight hyperbranched polyglycerols. Biomaterials 28:4779–4787
76. Kainthan RK, Hester SR, Levin E, Devine DV, Brooks DE (2007) In vitro biological evaluation of high molecular weight hyperbranched polyglycerols. Biomaterials 28:4581–4590
77. Kainthan RK, Janzen J, Levin E, Devine DV, Brooks DE (2006) Biocompatibility testing of branched and linear polyglycidol. Biomacromolecules 7:703–709
78. Kainthan RK, Mugabe C, Burt HM, Brooks DE (2008) Unimolecular micelles based on hydrophobically derivatized hyperbranched polyglycerols: ligand binding properties. Biomacromolecules 9:886–895
79. Calderón M, Quadir MA, Sharma SK, Haag R (2010) Dendritic polyglycerols for biomedical applications. Adv Mater 22:190–218
80. Calderón M, Reichert S, Welker P, Licha K, Kratz F, Haag R (2014) Receptor mediated cellular uptake of low molecular weight dendritic polyglycerols. J Biomed Nanotechnol 10: 92–99
81. Reichert S, Calderón M, Khandare J, Welker P, Mangoldt D, Licha K, Kainthan RK, Brooks DE, Haag R (2011) Size-dependant cellular uptake of dendritic polyglycerol. Small 7:820–829
82. Calderón M, Graeser R, Kratz F, Haag R (2009) Development of enzymatically cleavable prodrugs derived from dendritic polyglycerol. Bioorg Med Chem Lett 19:3725–3728
83. Calderón M, Welker P, Licha K, Graeser R, Kratz F, Haag R (2010) Development of efficient macromolecular prodrugs derived from dendritic polyglycerol. J Control Release 148:e21–e56
84. Calderón M, Welker P, Licha K, Fichtner I, Graeser R, Haag R, Kratz F (2011) Development of efficient acid cleavable multifunctional prodrugs derived from dendritic polyglycerol with a poly(ethylene glycol) shell. J Control Release 151:295–301
85. Hussain A, Krüger HR, Kampmeier F, Weissbach T, Licha K, Kratz F, Haag R, Calderón M, Barth S (2013) Targeted delivery of dendritic polyglycerol-doxorubicin conjugates by

scFv-SNAP fusion protein suppresses EGFR+ cancer cell growth. Biomacromolecules 14:2510–2520
86. Steinhilber D, Sisson AL, Mangoldt D, Welker P, Licha K, Haag R (2010) Synthesis, reductive cleavage, and cellular interaction studies of biodegradable, polyglycerol nanogels. Adv Funct Mater 20:4133–4138
87. Soloway AH, Tjarks W, Barnum BA, Rong F-G, Barth RF, Codogni IW, Wilson JG (1998) The chemistry of neutron capture therapy. Chem Rev 98:1515–1562
88. Hawthorne MF, Maderna A (1999) Applications of radiolabeled boron clusters to the diagnosis and treatment of cancer. Chem Rev 99:3421–3434
89. Nakanishi A, Guan L, Kane RR, Kasamatsu H, Hawthorne MF (1999) Toward a cancer therapy with boron-rich oligomeric phosphate diesters that target the cell nucleus. Proc Natl Acad Sci U S A 96:238–241
90. Barth RF, Adams DM, Soloway AH, Alam F, Darby MV (1994) Boronated starburst dendrimer-monoclonal antibody immunoconjugates: evaluation as a potential delivery system for neutron capture therapy. Bioconjug Chem 5:58–66
91. Capala J, Barth RF, Bendayan M, Lauzon M, Adams DM, Soloway AH, Robert A, Fenstermaker RA, Carlssonr J (1996) Boronated epidermal growth factor as a potential targeting agent for boron neutron capture therapy of brain tumors. Bioconjug Chem 7:7–15
92. Yang W, Barth RF, Adams DM, Soloway AH (1997) Intratumoral delivery of boronated epidermal growth factor for neutron capture therapy of brain tumors. Cancer Res 57:4333–4339
93. Wu G, Barth RF, Weilian Yang W, Chatterjee M, Tjarks W, Ciesielski MJ, Fenstermaker RA (2004) Site-specific conjugation of boron-containing dendrimers to anti-EGF receptor monoclonal antibody cetuximab (IMC-C225) and its evaluation as a potential delivery agent for neutron capture therapy. Bioconjug Chem 15:185–194
94. Qualmam B, Kessels MM, Musiol H-J, Sierralta WD, Jungblut PW, Moroder L (1996) Synthesis of boron-rich lysine dendrimers as protein labels in electron microscopy. Angew Chem Int Ed 35:909–911
95. Sheikhi Mehrabadi F, Fischer W, Haag R (2012) Dendritic and lipid-based carriers for gene/siRNA delivery (a review). Curr Opin Solid St M 16:310–322
96. Smith DK (2008) Dendrimers and the double helix-from DNA binding towards gene therapy. Curr Top Med Chem 8:1187–1203
97. Pandita D, Santos JL, Rodrigues J, Pêgo AP, Granja PL, Tomás H (2011) Gene delivery into mesenchymal stem cells: a biomimetic approach using RGD nanoclusters based on poly(amidoamine) dendrimers. Biomacromolecules 12:472–481
98. Al-Dosari MS, Gao X (2009) Nonviral gene delivery: principle, limitations, and recent progress. AAPS J 11:671–681
99. Russ V, Elfberg H, Thoma C, Kloeckner J, Ogris M, Wagner E (2008) Novel degradable oligoethylenimine-acrylate ester-based pseudo dendrimers for in vitro and in vivo gene transfer. Gene Ther 15:18–29
100. Russ V, Gunther M, Halama A, Ogris M, Wagner E (2008) Oligoethylenimine-grafted polypropylenimine dendrimers as degradable and biocompatible synthetic vectors for gene delivery. J Control Release 132:131–140
101. Fischer W, Calderón M, Schulz A, Andreou L, Weber M, Haag R (2010) Dendritic polyglycerols with oligoamine shells show low toxicity and high siRNA transfection efficiency in vitro. Bioconjug Chem 21:1744–1752
102. Waite CL, Roth CM (2009) PAMAM-RGD Conjugates enhance siRNA delivery through a multicellular spheroid model of malignant glioma. Bioconjug Chem 20:1908–1916
103. Kaneshiro TL, Lu ZR (2009) Targeted intracellular codelivery of chemotherapeutics and nucleic acid with a well-defined dendrimer-based nanoglobular carrier. Biomaterials 30: 5660–5666
104. Juliano RL, Ming X, Nakagawa O, Xu R, Yoo H (2011) Integrin targeted delivery of gene therapeutics. Theranostics 1:211–219
105. Kang C, Yuan X, Li F, Pu P, Yu S, Shen C, Zhang Z, Zhang Y (2009) Evaluation of folate-PAMAM for the delivery of antisense oligonucleotides to rat C6 glioma cells in vitro and in vivo. J Biomed Mater Res A 93:585–594

106. Lee H, Larson RG (2009) Molecular dynamics study of the structure and interparticle interactions of polyethylene glycol-conjugated PAMAM dendrimers. J Phys Chem B 113:13202–13207
107. Wang W, Xiong W, Wan J, Sun X, Xu H, Yang X (2009) The decrease of PAMAM dendrimer-induced cytotoxicity by PEGylation via attenuation of oxidative stress. Nanotechnology 20:105103
108. Guillaudeu SJ, Fox ME, Haidar YM, Dy EE, Szoka FC, Fréchet JMJ (2008) PEGylated dendrimers with core functionality for biological applications. Bioconjug Chem 19:461–469
109. Froehlich E, Mandeville JS, Jennings CJ, Sedaghat-Herati R, Tajmir-Riahi HA (2009) Dendrimers bind human serum albumin. J Phys Chem B 113:6986–6993
110. Kaminskas LM, Wu Z, Barlow N, Krippner GY, Boyd BJ, Porter CJ (2009) Partly-PEGylated poly-L-lysine dendrimers have reduced plasma stability and circulation times compared with fully PEGylated dendrimers. J Pharm Sci 98:3871–3875
111. Okuda T, Kawakami S, Akimoto N, Okuda T, Kawakami S, Akimoto N, Niidome T, Yamashita F, Hashida M (2006) PEGylated lysine dendrimers for tumor-selective targeting after intravenous injection in tumor-bearing mice. J Control Release 116:330–336
112. Qi R, Gao Y, Tang Y, He RR, Liu TL, He Y, Sun S, Li BY, Li YB, Liu G (2009) PEG-conjugated PAMAM dendrimers mediate efficient intramuscular gene expression. AAPS J 11:395–405
113. Taratula O, Garbuzenko OB, Kirkpatrick P, Pandya I, Savla R, Pozharova VP, He H, Minko T (2009) Surface-engineered targeted PPI dendrimer for efficient intracellular and intratumoral siRNA delivery. J Control Release 140:284–293
114. Ofek P, Fischer W, Calderón M, Haag R, Satchi-Fainaro R (2012) In vivo delivery of small interfering RNA to tumors and their vasculature by novel dendritic nanocarriers. FASEB J 24:3122–3134
115. Wan K, Ebert B, Voigt J, Haag R, Kemmer W (2012) In vivo tumor imaging using a novel RNAi-based detection mechanism. Nanomedicine 8:393–398
116. Eyal S, Hsiao P, Unadkat JD (2009) Drug interactions at blood-brain barrier: fact or fantasy? Pharmacol Ther 123:80–104
117. Alam MI, Beg S, Samad A, Baboota S, Kohli K, Ali J, Ahuja A, Akbar M (2010) Strategy for effective brain drug delivery. Eur J Pharm Sci 5:385–403
118. Jones AR, Shusta EV (2009) Blood-brain barrier transport of therapeutics via receptor-mediation. Pharm Res 24:1759–1771
119. Pardridge W (1995) Transport of small molecules through the blood- brain barrier: biology and methodology. Adv Drug Deliv Rev 15:5–36
120. Terasaki T, Hosoya K (1999) The blood-brain barrier efflux transporters as a detoxifying system for the brain. Adv Drug Deliv Rev 36:195–209
121. Suzuki H, Terasaki T, Sugiyama Y (1997) Role of efflux transport across the blood-brain barrier and blood–cerebrospinal fluid barrier on the disposition of xenobiotics in the CNS. Adv Drug Deliv Rev 25:257–285
122. Tsuji A, Tamai I (1999) Carrier-mediated or specialized transport of drugs across the blood-brain barrier. Adv Drug Deliv Rev 36:277–290
123. Kusuhara H, Sugiyama Y (2001) Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (Part 1). Drug Discov Today 6:150–156
124. Pardridge WM (2001) Crossing the blood–brain barrier: are we getting it right? Drug Discov Today 6:1–2
125. Pardridge WM (2002) The lack of BBB research. Drug Discov Today 7:223–226
126. Kaiser S, Toborek M (2001) Liposome-mediated high-efficiency transfection of human endothelial cells. J Vasc Res 2:133–143
127. Torchilin VP, Levchenko TS, Rammohan R, Volodina N, Papahadjopoulos-Sternberg B, D′Souza GGM (2003) Cell transfection in vitro and in vivo with nontoxic tat peptide-liposome-DNA complexes. Proc Natl Acad Sci U S A 100:1972–1977
128. Bartlett JS, Wilcher R, Samulski RJ (2000) Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. J Virol 74:2777–2785

129. Zhou J, Wu J, Hafdi N, Behr JP, Erbacher P, Peng L (2006) PAMAM dendrimers for efficient siRNA delivery and potent gene silencing. Chem Commun (Camb) 22:2362–2364
130. Lechardeur D, Verkman AS, Lukacs GL (2005) Intracellular routing of plasmid DNA during non-viral gene transfer. Adv Drug Deliv Rev 57:755–767
131. Ke W, Shao K, Huang R, Han L, Liu Y, Li J, Kuang Y, Ye L, Lou J, Jiang C (2009) Gene delivery targeted to the brain using an Angiopep-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. Biomaterials 30:6976–6985
132. Ying X, Wen H, Lu W, Du J, Guo J, Tian W, Mena Y, Zhang Y, Li R-J, Yang T-Y, Shang D-W, Lou J-N, Zhang L-R, Zhang Q (2010) Dual-targeting daunorubicin liposomes improve the therapeutic efficacy of brain glioma in animals. J Control Release 141:183–192
133. Carroll RT, Bhatia D, Geldenhuys W, Bhatia R, Miladore N, Bishayee A, Sutariya V (2010) Brain-targeted delivery of Tempol-loaded nanoparticles for neurological disorders. J Drug Target 18:665–674
134. Wu G, Barth RF, Yang W, Kawabata S, Zhang L, Green-Church K (2006) Targeted delivery of methotrexate to epidermal growth factor receptor-positive brain tumors by means of cetuximab (IMC-C225) dendrimer bioconjugates. Mol Cancer Ther 25:52–59
135. Ren Y, Kang CS, Yuan XB, Zhou X, Xu P, Han L, Wang GX, Jia Z, Zhong Y, Yu S, Sheng J, Pu PY (2010) Co-delivery of as-miR-21and 5-FU by poly(amidoamine) dendrimer attenuates human glioma cell growth in vitro. J Biomater Sci Polym Ed 21:303–314
136. Visser CC, Voorwinden LH, Crommelin DJ, Danhof M, de Boer AG (2004) Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. Pharm Res 5:761–769
137. Pardridge WM (2005) Tyrosine hydroxylase replacement in experimental Parkinson's disease with transvascular gene therapy. NeuroRx 2:129–138
138. Blasi P, Giovagnoli S, Schoubben A, Ricci M, Rossi C (2007) Solid lipid nanoparticles for targeted brain drug delivery. Adv Drug Deliv Rev 59:454–477
139. Gajbhiye V, Jain NK (2011) The treatment of glioblastoma xenografts by surfactant conjugated dendritic nanoconjugates. Biomaterials 32:6213–6225
140. Pardridge WM (2002) Drug and gene targeting to the brain with molecular Trojan horses. Nat Rev Drug Discov 2:131–139
141. Li HY, Qian ZM (2002) Transferrin/transferrin receptoremediated drug delivery. Med Res Rev 3:225–250
142. Jefferies WA, Brandon MR, Hunt SV, Williams AF, Gatter KC, Mason DY (1984) Transferrin receptor on endothelium of brain capillaries. Nature 312:162–163
143. Hall WA (1991) Transferrin receptor on glioblastoma multiforme. J Neurosurg 74:313–314
144. Ferretti C, Blengio M, Ghi P, Racca S, Genazzani E, Portaleone P (1988) Tamoxifen counteracts estradiol induced effects on striatal and hypophyseal dopamine receptors. Life Sci 42:2457–2465
145. Kayyali R, Marriott C, Wiseman H (1994) Tamoxifen decreases drug efflux from liposomes: relevance to its ability to reverse multidrug resistance in cancer cells? FEBS Lett 344: 221–224
146. Li Y, He H, Jia X, Lu WL, Lou J, Wei Y (2012) A dual-targeting nanocarrier based on poly(amidoamine) dendrimers conjugated with transferrin and tamoxifen for treating brain gliomas. Biomaterials 33:3899–3908
147. Dai H, Navath RS, Balakrishnan B, Guru BR, Mishra MK, Romero R, Kannan RM, Kannan S (2010) Intrinsic targeting of inflame matory cells in the brain by polyamidoamine dendrimers upon subarachnoid administration. Nanomedicine (Lond) 5:1317–1329
148. Qian ZM, Li H, Sun H, Ho K (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54(4):561–587
149. Hatakeyama H, Akita H, Maruyama K, Suharac T, Harashima H (2004) Factors governing the in vivo tissue uptake of transferrin-coupled polyethylene glycol liposomes in vivo. Int J Pharm 281:25–33
150. Huang R, Ke W, Han L, Liu Y, Shao K, Ye L, Lou J, Jiang C, Pei Y (2009) Brain-targeting mechanisms of lactoferrin-modified DNA-loaded nanoparticles. J Cereb Blood Flow Metab 29:1914–1923

151. Huang R, Ke W, Liu Y, Jiang C, Pei Y (2008) The use of lactoferrin as a ligand for targeting the polyamidoamine-based gene delivery system to the brain. Biomaterials 29:238–246
152. Sarin H, Kanevsky AS, Wu H, Brimacombe KR, Fung SH, Sousa AA, Auh S, Wilson CM, Sharma K, Aronova MA, Leapman RD, Griffiths GL, Hall MD (2008) Effective transvascular delivery of nanoparticles across the blood-brain tumor barrier into malignant glioma cells. J Transl Med 6:80
153. Cevc G (2004) Lipid vesicles and other colloids as drug carriers on the skin. Adv Drug Deliv Rev 56:675–711
154. Choi MJ, Maibach HI (2005) Liposomes and niosomes as topical drug delivery systems. Skin Pharmacol Physiol 18:209–219
155. Schäfer-Korting M, Mehnert W, Korting HC (2007) Lipid nanoparticles for improved topical application of drugs for skin diseases. Adv Drug Deliv Rev 59:427–443
156. Müller RH, Radtke M, Wissing SA (2002) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv Drug Deliv Rev 54(Suppl 1):S131–S155
157. Kogan A, Garti N (2006) Microemulsions as transdermal drug delivery vehicles. Adv Colloid Interface Sci 123–126:369–385
158. Lopes LB, Ferreira DA, de Paula D, Garcia MT, Thomazini JA, Fantini MC, Bentley MV (2006) Reverse hexagonal phase nanodispersion of monoolein and oleic acid for topical delivery of peptides: in vitro and in vivo skin penetration of cyclosporin A. Pharm Res 23:1332–1342
159. Bouwstra JA, Honeywell-Nguyen PL (2002) Skin structure and mode of action of vesicles. Adv Drug Deliv Rev 54(Suppl 1):S41–S55
160. Shi X, Lee I, Chen X, Shen M, Xiao S, Zhu M, Baker JR Jr, Wang SH (2010) Influence of dendrimer surface charge on the bioactivity of 2-methoxyestradiol complexed with dendrimers. Soft Matter 6:2539–2545
161. Sun M, Fan A, Wang Z, Zhao Y (2012) Dendrimer-mediated drug delivery to the skin. Soft Matter 8:4301–4305
162. Yang Y, Sunoqrot S, Stowell C, Yang Y, Sunoqrot S, Stowell C, Ji J, Lee C-W, Kim JW, Khan SA, Hong S (2012) Effect of size, surface charge, and hydrophobicity of poly(amidoamine) dendrimers on their skin penetration. Biomacromolecules 13:2154–2162
163. Venuganti VVK, Perumal OP (2009) Poly(amidoamine) dendrimers as skin penetration enhancers: influence of charge, generation, and concentration. J Pharm Sci 98:2345–2356
164. Borowskaa K, Wołowieca S, Rubaj A, Głowniakc K, Sieniawskac E, Radej S (2012) Effect of polyamidoamine dendrimer G3 and G4 on skin permeation of 8-methoxypsoralene-In vivo study. Int J Pharm 426:280–283
165. Haag R (2004) Supramolecular drug-delivery systems based on polymeric core-shell architectures. Angew Chem Int Ed 43:278–282
166. Radowski MR, Shukla A, von Berlepsch H, Böttcher C, Pickaert G, Rehage H, Haag R (2007) Supramolecular aggregates of dendritic multishell architectures as universal nanocarriers. Angew Chem Int Ed 46:1265–1269
167. Küchler S, Radowski MR, Blaschke T, Dathe M, Plendl J, Haag R, Schäfer-Korting M, Kramer KD (2009) Nanoparticles for skin penetration enhancement-A comparison of a dendritic core-multishell-nanotransporter and solid lipid nanoparticles. Eur J Pharm Biopharm 71:243–250
168. Küchler S, Abdel-Mottaleb M, Lamprecht A, Radowski MR, Haag R, Schäfer-Korting M (2009) Influence of nanocarrier type and size on skin delivery of hydrophilic agents. Int J Pharm 377:169–172
169. Iezzi R, Guruc BR, Glybinaa IV, Mishra MK, Kennedy A, Kannan RM (2012) Dendrimer-based targeted intravitreal therapy for sustained attenuation of neuroinflammation in retinal degeneration. Biomaterials 33:979–988
170. Chandrasekar D, Sistla R, Ahmad FJ, Khar RK, Diwan PV (2007) Folate coupled poly(ethyleneglycol) conjugates of anionic poly(amidoamine) dendrimer for inflammatory tissue specific drug delivery. J Biomed Mater Res A 82A:92–103

171. Hayder M, Poupot M, Baron M, Nigon D, Turrin CO, Caminade AM, Majoral JP, Eisenberg RA, Fournié JJ, Cantagrel A, Poupot R, Davignon JL (2011) A phosphorus-based dendrimer targets inflammation and osteoclastogenesis in experimental arthritis. Sci Transl Med 3:81ra35
172. Bosch X (2011) Dendrimers to treat rheumatoid arthritis. ACS Nano 5:6779–6785
173. Shaunak S, Thomas S, Gianasi E, Godwin A, Jones E, Teo I, Mireskandari K, Luthert P, Duncan R, Patterson S, Khaw P, Brocchini S (2004) Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation. Nat Biotechnol 22:977–984
174. Thomas TP, Goonewardena SN, Majoros IJ, Kotlyar A, Cao Z, Leroueil PR, Baker JR Jr (2011) Folate-targeted nanoparticles show efficacy in the treatment of inflammatory arthritis. Arthritis Rheum 63:2671–2680
175. Dernedde J, Rausch A, Weinhart M, Enders S, Tauber R, Licha K, Schirner M, Zügel U, von Bonin A, Haag R (2010) Dendritic polyglycerol sulfates as multivalent inhibitors of inflammation. Proc Natl Acad Sci U S A 107:19679–19684
176. Licha K, Welker P, Weinhart M, Wegner N, Kern S, Reichert S, Gemeinhardt I, Weissbach C, Ebert B, Haag R, Schirner M (2011) Fluorescence imaging with multifunctional polyglycerol sulfates: novel polymeric near-IR probes targeting inflammation. Bioconjug Chem 22: 2453–2460
177. Fasting C, Schalley C, Weber M, Seitz O, Hecht S, Koksch B, Dernedde J, Graf C, Knapp E-W, Haag R (2012) Multivalency as a chemical organization and action principle. Angew Chem Int Ed 51:10472–10498

Chapter 18
Inorganic Nanoparticles in Targeted Drug Delivery and Imaging

Hélder A. Santos, Luis M. Bimbo, Leena Peltonen, and Jouni Hirvonen

18.1 Introduction

The field of nanotechnology has opened up new avenues for design, fabrication, and production of nanomaterials with superior properties that can be useful for biomedical applications. The fast growing health problems in the world and societies demand rapid development of nanomedicines for fighting against different diseases. Current pharmaceuticals are limited in operating efficiently due to their physicochemical shortcomings in the delivery, biodistribution, and pharmacokinetics. Thus, nanomedicines are engineered as drug delivery carriers, while addressing the scientific challenges due to their nano-size.

Nanoparticles are usually synthesized from a vast number of raw materials, including gold, lipids, carbon, silica, silicon, iron, and other inorganic materials [1–5]. In this context, the field of inorganic nanomedicine has emerged in the preparation and characterization of nanoparticles with functionalities towards the applications in clinic. Research endeavors of inorganic nanomaterials were initially focused on physics, optics, and engineering, but have for quite some time now included also biological and medical applications. Often, the same nanomaterials can serve as biochemical sensors, contrast agents in cellular or tissue imaging, drug delivery vehicles, or even as therapeutics [6].

The understanding on the nanomaterials function(s) in a biological system depends on the interface between the biological system and nanomaterial—the nano–bio interface. This is especially true with regard to the size, shape, and surface chemistry. These parameters can be utilized to enable and tailor the development of nanotechnologies for chemical (bio)sensing, imaging, and therapeutic applications [7, 8]. In the comprehensive reviews by Ferrari [9] and Farokhzad and Langer [10]

H.A. Santos • L.M. Bimbo • L. Peltonen • J. Hirvonen (✉)
Division of Pharmaceutical Technology, University of Helsinki, Helsinki, Finland
e-mail: jouni.hirvonen@helsinki.fi

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_18

the possible benefits and outcomes of the improved therapeutic applications by nanotechnology have been summarized: (1) improved delivery of poorly water-soluble drugs; (2) targeted delivery of drugs in a cell- or tissue-specific manner; (3) transcytosis of drugs across epithelial and endothelial barriers; (4) delivery of large and macromolecule-type drugs to intracellular sites of action; (5) co-delivery of two or more drugs or therapeutic modalities for combination therapy; (6) visualization of the sites of drug delivery by combining therapeutic agents with imaging modalities (theranostics); and (7) real-time monitoring of the in vivo efficacy of the therapeutic agents.

Nanoparticles that are intended to enhance drug dissolution, such as nanocrystals and mesoporous materials, need to release the confined drug into the gastrointestinal (GI)-tract/intestinal wall, whereafter the free drug needs to pass through barriers similar to those of the conventional drug delivery formulations [8, 11–14]. On the other hand, nanoparticles encapsulating drugs which are intended to enter the bloodstream and targeted into various parts of the body, have to overcome further organ-, epithelium- and cell-specific barriers and survive the clearance mechanisms in vivo for sufficient duration to show the required efficacy [15]. The latter is often the case with the inorganic nanoparticles that have been studied widely for active and passive drug targeting purposes (Fig. 18.1).

The aim of targeted drug delivery is to obtain high local concentrations of drug together with low systemic exposure. Passive extravasation of nanoparticles takes place through the leaky vasculature, which is characteristic for solid tumors and inflamed tissues, via preferential accumulation through the enhanced permeability and retention (EPR) effect [16] (Fig. 18.1a). In this case, the drug is released in the extracellular matrix and diffuses throughout the tissue for bioactivity. Passive targeting lacks cell-specific interactions needed to induce nanocarrier internalization, thus decreasing the therapeutic efficacy which can result in multiple drug resistance [17]. Active targeting, on the other hand, enhances the therapeutic efficacy of drugs that do not permeate easily the cell membrane and require an intracellular site (target) for bioactivity, enhancing the overall drug bioavailability (Fig. 18.1b). Targeting nanoparticles can be engineered for both the approaches, leaky vasculature (e.g., tumors and inflammation) and target organs/cells (e.g., ligand binding to cell-surface receptors). Once the nanoparticles have extravasated in the target tissue, the targeting ligands on the particle surface can result in active targeting of the nanoparticles to specific receptors that are present on the target cells or tissues, resulting in enhanced accumulation and cell uptake through a receptor-mediated endocytosis. In this case there is a careful selection of selective targeting ligands that specifically interact with the receptors of the cell surface of interest to promote nanocarrier binding and internalization [18], which on the other hand requires that the receptors are highly overexpressed by cancer cells relative to normal cells.

The biomedical applications of inorganic nanoparticles have been extensively reviewed in the literature [4, 5, 19]. The major benefits associated with these systems are low toxicity and promise for controlled drug/gene delivery providing a true alternative to viral vectors and cationic carriers. Inorganic nanoparticles are generally versatile materials, including wide availability, rich functionality, good

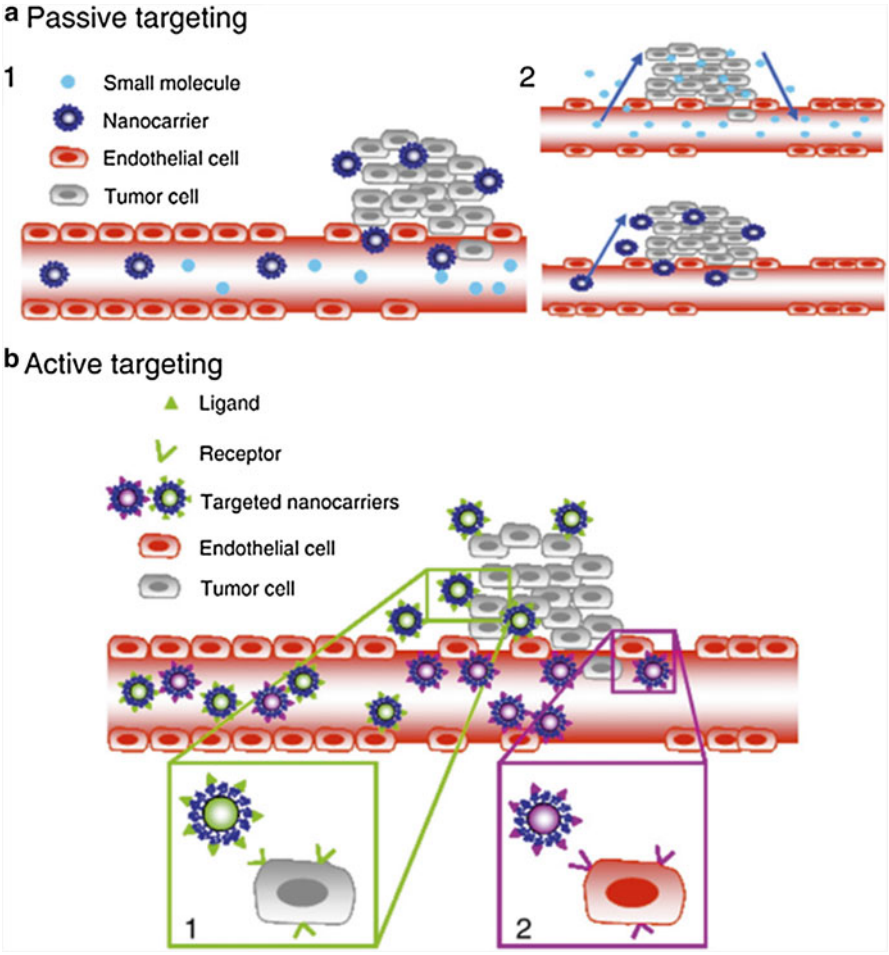

Fig. 18.1 Schematic representation of passive and active tumor targeting of nanocarriers. (**a**) Passive targeting: (1) Nanocarriers reach tumors selectively through the leaky vasculature surrounding the tumors; (2) Influence of the size for retention in the tumor tissues. Drugs alone diffuse freely in and out of the tumor blood vessels due to their small size, thus reducing their effective concentrations in the tumor, unlike drug-loaded nanocarriers that are larger in size and cannot diffuse back into the blood stream, enhancing the progressive accumulation in the tumor through the EPR effect. (**b**) Active targeting: Ligands attached at the surface of the nanocarriers bind to overexpressed receptors of the cancer cells (1) or angiogenic endothelial cells (2). Reproduced from [196]

biocompatibility, and potential for controlled release and targeted delivery of the carried drugs/genes. Oftentimes, however, the inorganic materials as such are not suitable for biomedical use. Therefore, hybrid nanomaterials and nanocomposites are warranted. Table 18.1 lists some of the major properties, benefits and drawbacks, and applications towards targeted drug delivery of inorganic nanoparticles and nanocomposites.

Table 18.1 Inorganic nanoparticles and nanocomposite materials for controlled and targeted drug delivery and imaging purposes [4, 5, 7, 8, 19–23]

Nanoparticle/ nanocomposite materials	Properties	Benefits	Drawbacks	Therapeutical goals
Mesoporous silicon/silica	Well-defined silanol groups on surface; suitable for chemical treatments and pore- and/or surface functionalization	Versatility, biocompatibility, low cytotoxicity; ease of functionalization	Dark color of porous silicon; surface chemistry dependent biosafety profiles	Dissolution enhancement; drug and gene delivery and targeting
Gold (Au)	Strong plasmon absorption and photothermal conversion of Au nanoparticles has been utilized in cancer therapy through selective localized photothermal heating of cancer cells	Easily tailored to a desired size range (1–200 nm); the surface can be modified for various functionalities, typically via thiols; good biocompatibility	Biosafety issues of Au	Targeted cellular delivery after conjugation; imaging applications; conjugated gene delivery vector with other nanomaterials
Silver (Ag)	Ag nanoparticles are promising materials for sustained delivery of Ag ions for bactericidal activity in wound- or burn-dressings	Photophysical characteristics and electrochemiluminescence of Ag nanoparticles; clusters are advantageous properties in molecular sensing applications	Biosafety issues of Ag	Nanosilver based wound dressings; biosensors; anti-HIV activity
Super-paramagnetic iron oxide	Magnetite (Fe_3O_4) and maghemite (Fe_2O_3) have been studied and used for targeted drug delivery using strong magnetic fields	Precisely targeted delivery at a specific site; versatile manufacture and shape as nanowires, nanospheres, nanotubes and magnetic thin films	Need for a strong external magnetic field	Drug delivery and targeting; bioimaging; hyperthermal treatment of tumors
Quantum dots (QDs)	QDs are inorganic nanocrystals (2–10 nm) that possess unique luminescent properties; semiconductor core overcoated by a shell to improve optical properties, and a cap-improving aqueous solubility	Often used as fluorescent labels with better brightness and resistance against photobleaching, with multicolor fluorescence emission	Derivatization and ligands needed for hydrophilicity	Potential intravascular probes for both diagnostics (imaging) and therapeutics (drug delivery); tumor targeting and imaging

Gadolinium oxide	Shape of the nanocrystals readily controlled by tailoring reaction parameters such as temperature and time	Highly uniform Gd_2O_3 nanoplates self-assemble into nanofibril-like liquid-crystalline superlattices with long-range orientational and positional order; strong paramagnetic response for a potential magnetic resonance imaging contrast agent	Size and shape dependency of biological interactions; monodispersity requirement for the reliable biological interaction measurements	Multimodal bioimaging probes
Layered double hydroxide (LDH)	Natural or synthetic anionic exchanging clays MII MIII (OH) Am·H_2O (MII = Mg, Zn, Ca, Co, Fe, Ni, Cu…; MIII = Al, Fe, Cr, Ga…; Am = Cl, CO_2, NO…); e.g., hydrotalcite $Mg_6Al_2(OH)_{16}CO_3{\cdot}4H_2O$	Tailored nanomaterials (30–100 nm) with a high zeta potential (+45 mV); hydrophilicity, biodegradability biocompatibility and relatively low toxicity; release controlled by pH adjustment		Drug delivery and targeting; effective non-viral gene delivery agents
Fullerenes	Hydrophobic 1-nm scale carbon spheres; may be functionalized by attaching hydrophilic moieties to improve aqueous solubility properties	Derivatized fullerenes are able to cross cell membrane and, e.g., bound to mitochondria		Carriers for genes and proteins; drug targeting
Carbon nanotubes (CNTs)	Hydrophobic CNTs can be modified water-soluble nanotubes; peptide-conjugated CNTs can move across cell membranes and even reach the nucleus of the cell	Good biocompatibility, low cytotoxicity, unique physicochemical properties	Needle-like shape of the CNTs, possible asbestos-like effects; low drug delivery capacity inside the CNTs	Drug delivery and targeting
Graphene	Nano-graphene oxide materials are single-layer graphene oxide sheets of a few nanometers in lateral width	Electronics, membranes, and nanocomposites applications; large specific surface area; nanohybrids and nanocomposites	Graphene aggregates easily in biological fluids; chemical functionalization needed to improve solubility and compatibility in biological environments	Drug delivery and targeting; live cell imaging
Dendrimers	Globular nanostructures specifically engineered to carry molecules encapsulated in the interior void spaces or attached to the surface structures	Size, shape, and reactivity are determined by generation (shells), chemical composition of the core, interior branching, and surface functionalities; truly customizable nanomaterials	Multicomposite systems with expertise requested in manufacturing	Drug or gene carriers; contrast agents and sensors for different metal ions

Table 18.1. Inorganic nanoparticles and nanocomposite materials for controlled and targeted drug delivery and imaging purposes [4, 5, 7, 8, 19–23].

Fine metal and other inorganic particles with nanometer-scale dimensions are of great interest due to their favorable functional properties. For example, nanometer-scale metallic wires show combinations of high strength, stiffness, and ductility. Therefore, nanorods and nanowires have found promising applications in electronics, photonics, hierarchical biology inspired nanocomposites, (bio)chemical sensing and imaging, and drug delivery/targeting [24]. Gold colloids have been widely used as contrast agents in electron microscopy because of their high electron-density [25]. Also, gold and silver nanoparticles have been used in biological optical imaging and sensing applications due to the excellent elastic light scattering properties [24, 26, 27]. Furthermore, gold nanoshells of appropriate size and shape absorb large amounts of near-infrared (NIR) light that can be conducted into the surrounding tissues as heat, which might be utilized as a novel cancer therapy towards cancer cells [28].

As stated above, the uptake of nanoparticles by living cells is affected by the properties of the nanoparticles, such as size, shape and surface. However, nanoparticles dispersed in a biological fluid are rapidly covered by biomolecules (proteins and lipids) forming a biomolecular corona that effectively covers the bare nanoparticle's surface [29]. When cells are exposed to nanoparticles it is, therefore, typical that the nanoparticle–biomolecular corona complexes interact with the components of the cell membrane. This is followed by the activation of an energy-dependent uptake mechanism, which allows the nanoparticles to be internalized into the cell and further trafficked to different subcellular locations, typically ending in lysosomal accumulation. Biocompatibility and biosafety are, therefore, crucial parameters for the development of inorganic nanomaterials towards therapeutical applications.

In this chapter, the focus is on the delivery, targeting, and imaging aspects of inorganic nanoparticles. Major discussion is allocated to: (1) mesoporous silicon- and silica-based materials; (2) metal nanoparticles, including theranostic applications of colloidal gold and silver; (3) superparamagnetic iron oxide nanoparticles as candidates for drug targeting and magnetic resonance imaging (MRI) and biological separation combined to localized magnetic heating effect in the presence of a strong magnetic field; and (4) quantum dots (QDs) formed of inorganic semiconductor materials such as CdSe. All these nanomaterials can be further functionalized to reach optimal delivery, targeting, and safety profiles for the intended therapeutical purpose, e.g., cancer therapy. As structurally and functionally carbon-based materials, fullerene-, graphene-, carbon nanotube-, and dendrimer-based nanohybrids and nanocomposites have been left out from detailed descriptions in this chapter of inorganic nanoparticles.

18.2 Mesoporous Silica-and Silicon-Based Systems

The development of mesoporous (defined by IUPAC as pores with diameters between 2 and 50 nm) silica and silicon materials for biomedical applications has been explored for a couple of decades now, since the discovery by Mobil researchers

for the mesoporous silica materials and by Leigh Canham for the mesoporous silicon in early 1990s [30, 31]. The ability to design relatively uniformly sized porous and dispersible nanoparticles using colloidal chemistry and evaporation-induced self-assembly (mesoporous silica) and electrochemistry etching (mesoporous silicon) has led to many applications of these mesoporous materials for delivery of therapeutics to the cells and for diagnostic and bioimaging applications [32–40]. These materials are relatively stable to heat, pH, and mechanical stress. The nanoparticles can protect the cargo until it gets released inside the cell.

The exceptionally high surface area of the mesoporous silica and silicon materials that can exceed 1,000 m^2/g and 500 m^2/g, respectively, and the possibility to independently modify the pore size and surface chemistry enables the loading of diverse single or combinations of payloads at levels exceeding those of other common drug delivery carriers, such as liposomes or polymer conjugates. This is mainly due to the physisorption of drugs resulting from the non-covalent electrostatic, hydrogen-bonding, and van der Waals interactions between the payloads and the internal surface of the mesopores, which allow loading capacities that surpass the solubility limit of the payloads in solution. In addition, the possibility to independently modify the external and interior surface of the mesoporous materials enables further engineered biofunctionality and biocompatibility of these materials.

18.2.1 Fabrication Procedures

18.2.1.1 Mesoporous Silica

Since the first report in 2001 describing the use of MCM-41 (Mobil Composition of Matter No. 41) type mesoporous silica nanoparticles as drug delivery system [41], we have seen an exponential growth in the literature of works covering applications of mesoporous silica in biological systems. For targeted intracellular delivery the particle sizes of interest range from 50 to 200 nm, because these are small enough particles that can bypass physiological membranes in the body. On the other hand, it is not feasible to synthesize smaller mesoporous silica nanoparticles due to their inherent mesoporosity. Thus, wet chemical synthesis methods are generally more beneficial than other physical methods.

In general, the fabrication routes of mesoporous silica-based materials are modified versions of the synthesis of MCM-41, by far the most extensively studied mesoporous material with ordered hexagonally arranged cylindrical mesopores [31, 42]. In the case of MCM-41 the synthesis occurs in aqueous solution, where above the critical micelle concentration, amphiphilic surfactant molecules self-assemble into spherical micelles and at higher concentrations into periodic liquid crystal mesophases. In the presence of water and hydrophilic, soluble silica precursors (e.g., silicic acid, $Si(OH)_4$, and polysilicic acids), the surfactant self-assembly results in hybrid nanocomposites. The silica precursors are then concentrated at the hydrophilic interfaces via electrostatic and hydrogen bonding interactions, and later

condensed to form amorphous silica of ordered periodic mesophase. The subsequent removal of the surfactant template by extraction or calcination results in the mesoporous product.

In general, the syntheses of such mesoporous silica-based materials result from the cooperative self-assembly of supramolecular aggregates of alkylammonium surfactants (commonly cetyl trimethylammonium bromide (CTAB)) with polymerizing silica species, e.g., precursor tetraethoxysilane (TEOS), under alkaline conditions [43, 44]. As a result, an organic–inorganic composite material consisting of hexagonally ordered rod-shaped micelles surrounded by an amorphous silica (SiO_2) network is formed. After removal of the organic portion by either thermal treatment or solvent extraction (ion exchange) inorganic (ceramic) materials with hexagonal, cylindrical, or ordered pores of typically 3–4 nm in diameter are produced [31, 42]. The advantage of this procedure is that the pore size of the material can be tuned by, for example, changing the surfactant, the surfactant chain length, or simply by adjusting the synthesis conditions. In Fig. 18.2 is shown an example of the synthesis and functionalization processes of mesoporous silica nanoparticles.

In order to avoid secondary aggregation of the particles and to easily control the spherical morphology of the silica materials, the syntheses are performed under either dilute acidic and neutral conditions [45, 46], or by further addition of particle growth quenchers [21, 43]. The former method is very reproducible producing monodispersed particles; however, the scaling up in large quantities is rather limited. The removal of the template is crucial in order to obtain well-dispersed nanoparticles. The surfactant removal is commonly done by solvent extraction.

In biological applications, several surface modifications to mesoporous silica can be employed, such as chemical modification, co-condensation, post-synthetic grafting, and surface coating. In chemical modification, the functionality is achieved by modifying silanol groups present both inside the pores and on the outer surface. Different chemical moieties can be introduced by adsorption onto the mesoporous

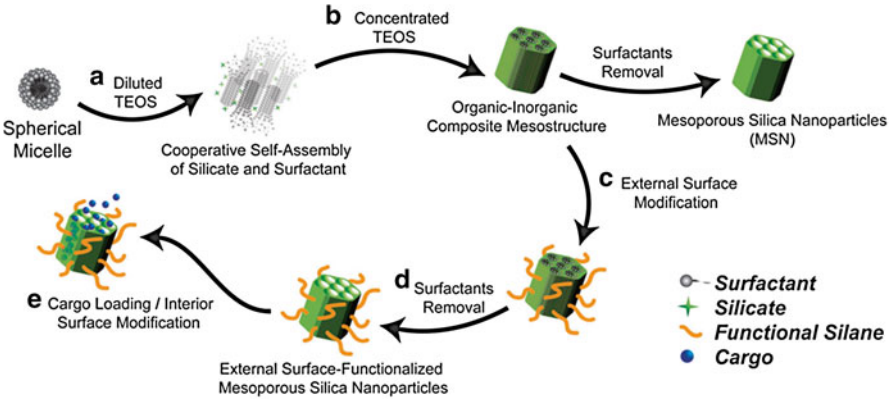

Fig. 18.2 An example for the synthesis and selective functionalization of mesoporous silica nanoparticles. Reproduced from [44]

material as a result of the attractive electrostatic interactions between the chemical moieties and the negatively charged surface of the carriers. In the case of co-condensation, hydrolyzed alkoxysilanes with organoalkoxysilanes are used to modify the inner pore surface [44]. After the silica condensation, the organoalkoxysilane also co-condenses positioning the chemical moiety onto the pore walls. In the case of post-synthetic grafting, the mesoporous material is modified after synthesis. Here, the surface-accessible silanol groups both in the mesopore network and on the external surfaces are modified. The former is achieved via condensation with trifunctional organosilanes in an organic solvent and it produces a self-assembled monolayer [47]. Another method to functionalize the mesoporous silica is by surface coating, where the functional groups are introduced directly on the external surface of the particles. These groups can then be reacted as linkers to attach other larger molecules or used to adsorb coatings through noncovalent interactions [48].

In order to use the mesoporous silica for targeting applications, cell-specific targeting ligands are covalently attached to the outer surface of nanoparticles, usually using the condensation method, thus enhancing the particles' cell-specificity. When the targeting moiety is a rather small molecular ligand (e.g., sugars and folic acid) the attachment can be done in organic or aqueous solvents before drug loading. However, for protein-based targeting moieties (e.g., polypeptides and antibodies), the conjugation step is preferably performed in an aqueous solvent to protect the proteins from possible premature degradation. In this case, the drug should not leak out during the conjugation step. In most cases, a simple bioconjugation reaction based on 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide or *N*,*N*′-dicyclohexylcarbodiimide are often used in the attachment. In addition, different fluorochromes can also be attached to the surface or deeper in the particle structure of the particles using a similar reaction often via an amine-specific reaction. When certain responsive polymers or PEG terminal functional groups are required, the targeting ligand can be attached to the polymer chains before conjugated to the particles. Since the ligand–receptor binding is conformation defined, it is of utmost importance that the ligand is attached to the particle in such a way that it retains its binding.

18.2.1.2 Mesoporous Silicon

As described above, the synthesis of mesoporous silica takes place through a bottom-up approach, whereas mesoporous silicon is produced via a top-down approach where the fabrication of size-controlled nanoparticles is usually done by mechanical size reduction using ultrasonication or milling. As in the case of mesoporous silica, also for mesoporous silicon materials several methods of fabrication have been reported in the literature [7, 8]. From these methods, the electrochemical anodization of monocrystalline Si-wafers in aqueous hydrofluoric acid (HF)-ethanolic electrolyte solution is the most frequent method for the production of mesoporous silicon [49]. Basically, in this simple method, the Si-wafer (anode) and a platinum (Pt) plate (cathode) are immersed in an electrolyte solution under an

applied potentiostatic (voltage-controlled) or galvanostatic (current-controlled) mode [7]. In such an electropolishing process, high anodic currents are produced to smooth the Si surfaces, whereas the pores are etched on the surface of the Si producing a porous layer at low anodic currents. The fabrication conditions (e.g., current density, wafer type and resistivity, HF concentration, chemical composition of the electrolyte, crystallographic orientation, temperature, duration of anodization and illumination) when operating below the electropolishing current threshold are crucial to control the properties of the mesoporous silicon materials, such as porosity, porous layer thickness, pore size and shape [50]. As a result, by applying an electropolishing current the lift-off of the porous layer from the bulk Si as a free-standing thin film can also be achieved (Fig. 18.3).

The production of the nanoparticles through mechanical size reduction starts with the fabrication of mesoporous silicon thin film. At this stage, the porous thin film may be constituted from a single uniform layer etched with a constant current, or from a multilayer stack, where periodically repeating high porosity layers function as fracture planes [51, 52]. The size reduction can be achieved by both milling and sonication and the initial thin films may also be chemically modified prior to the size reduction. In the case of photolithographic methods in the fabrication of mesoporous silicon nanoparticles, a considerably more control over the final particle size and shape can be achieved [53, 54].

After the anodization, the native surface of the mesoporous silicon materials is hydrogen terminated (Si–Hx), which is a rather unstable surface which undergoes slow, spontaneous oxidation in the presence of oxygen, water molecules or even in dry ambient air. In order to replace the unstable hydrogen termination of the freshly etched surfaces, various surface stabilization methods are employed. These methods produce higher stability based on oxidation, carbonization, hydrosilylation,

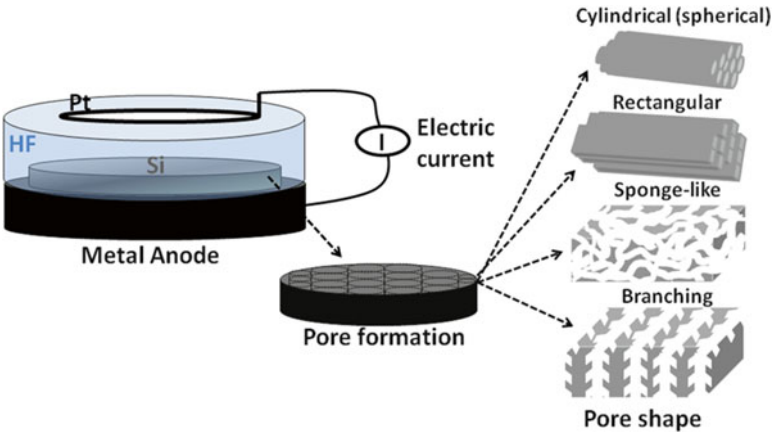

Fig. 18.3 Schematic representation of an electrochemical etching setup for mesoporous fabrication and pore formation: cylindrical (*spherical*) and rectangular without interconnections, sponge-like, and branching

and silanization of the mesoporous silicon surface, which can withstand the harsh physiological conditions for extended durations. These stabilization methods of the mesoporous silicon materials have been reported and reviewed in detail elsewhere [7, 11, 50, 52, 55–63].

In order to use the mesoporous silicon materials for targeting applications, the hydrosilylation method is often used to functionalize the surface of the materials, obtaining a specific surface termination, such as a carboxylic acid group for biomolecule conjugation [64, 65]. For example, by adapting the thermal hydrosilylation method for undecylenic acid with thermally hydrocarbonized mesoporous silicon particles, a –COOH terminated stable surface for controlled delivery of peptides has been obtained [66]. In addition, methods utilizing silane coupling chemistry more commonly used with mesoporous silica-based materials have also been employed with mesoporous silicon nanomaterials. The attachment of organofunctional molecules through siloxane (Si–O–Si) bridges on silanol terminated mesoporous silica or oxidized mesoporous silicon is a fairly straightforward process made in liquid or by vapor deposition [67, 68]. A common organosilane, (3-aminopropyl)triethoxysilane, has been reported on oxidized and carbonized mesoporous silicon [69–71], providing a positive surface charge to the surface of the materials due to the abundance of terminal amine groups, which in turn can be used for further bioconjugation processes [53, 71]. A similar surface biofunctionalization and cargos as described for mesoporous silica can also be employed in the case of mesoporous silicon nanoparticles. However, the studies described in the literature on the surface functionalization and targeting of mesoporous silicon are still scarce.

18.2.2 Targeting

Although there have been great advances in research in the last few decades, cancer therapy still suffers from a major challenge associated to the low therapeutic concentration of the drugs reaching the subcellular compartments of a target tissue, resulting from the lack of target selectivity [10]. To develop an effective therapeutic system with a higher probability of extravasation requests to fabricate the targeted particles with a size defined in the range of nanometer in order to avoid unwanted side-effects by the anticancer drug on healthy cells. Among the outstanding advantages of porous nanomaterials, the ability of surface functionalization with targeting moieties is the most exciting favorable result reported in the literature, which works as caps for sustained release of various cargos to cancerous cells and highlights the paramount importance of porous materials as a relevant platform for a wide range of pharmaceutical compounds. Both mesoporous silica and silicon can be employed for direct imaging and targeting under biorelevant conditions to follow the biodistribution, cancer cell targeting efficiency, internalization pathways, cytotoxicity, and the progress of therapy. However, in the following sections we mainly focus on the targeting properties of these materials.

18.2.2.1 Mesoporous Silica

As discussed previously, in order to be effective for biomedical applications, nanocarriers have to simultaneously present multiple functionalities and properties, such as: (1) ease of imaging; (2) dispersibility; (3) specificity; (4) ability to load and deliver large concentrations of diverse cargos; and (5) biocompatibility and low toxicity. In this context, the large surface area associated with a versatile surface chemistry and low toxicity render mesoporous silica nanomaterials ideal candidates for nanocarrier platforms. Mesoporous silica can be used for direct imaging and targeting. Biorelevant conditions allow the follow-up of biodistribution, cancer cell targeting efficiency, internalization pathways, cytotoxicity, and progress of therapy. In order to limit the degree of nonspecific binding while enhancing specific internalization by the target cell or tissue, mesoporous silica can be actively targeted towards the intended region.

Several studies have been reported in the literature for passive and active targeting using functionalized mesoporous silica nanoparticles. For example, Xia and coworkers have shown that mesoporous silica nanoparticles coated with polyethyleneimine (PEI) significantly enhanced the particle uptake [72]. A precise control of the size of the particles and of the PEI/PEG copolymer coating onto the surface of the particles was also shown to enhance the EPR effect and reduce the RES uptake on a xenograft model [73]. Coating of 50 nm mesoporous silica nanoparticles with PEI/PEG copolymer reached a high passive accumulation of about 12 % at the tumor site, compared with 1 % of 100 nm phosphonate-coated mesoporous silica nanoparticles and 3 % of 50 nm PEGylated mesoporous silica nanoparticles (Fig. 18.4a). The increased tumor accumulation further brought an enhanced tumor inhibition rate (Fig. 18.4b). In addition, the cationic polymer coated

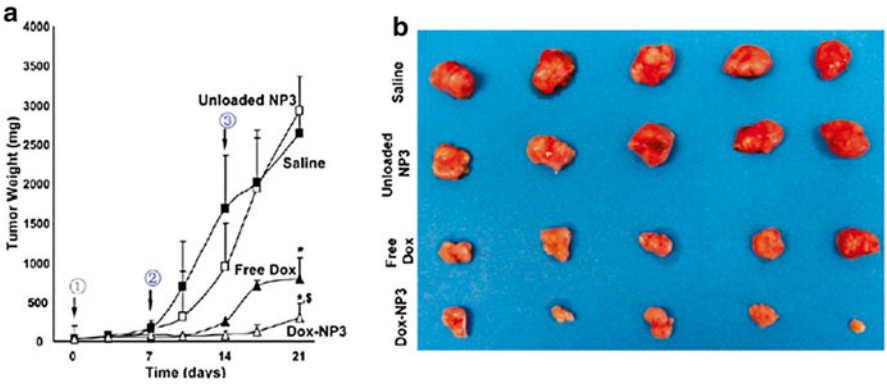

Fig. 18.4 (**a**) Comparison of the tumor inhibition effect of doxorubicin-loaded nanoparticles (Dox-NP3) vs. free drug (free Dox), empty particles, and saline in the KB-31 xenograft model. The tumor-bearing mice were intravenously injected with 120 mg/kg Dox-loaded NP3 weekly for 3 weeks. (**b**) Corresponding photographs of the tumor tissues at the end of therapy. Reproduced from [73]

on the surface of mesoporous silica nanoparticles increased the steric hindrance between nanoparticles improving the particle stability. Despite the promising results, it is still challenging to further increase the in vivo circulation time and the passive targeting efficiency for mesoporous silica-based drug delivery systems.

For the active targeting, several ligands (e.g., folate, arginine–glycine–aspartic acid (RGD) peptide, and transferrin) that bind specifically to receptors overexpressed on the cancer cells ligands, have been used to modify the surface of the nanoparticles [40, 74–78]. In order to be effective with highly specific binding affinity, a high concentration of the surface-conjugated ligands is required in order to promote multivalent binding effects. Thus, a more efficient drug delivery through receptor-mediated internalization pathways can be achieved.

Recently, Brinker and coworkers fused supported lipid bilayer onto mesoporous silica nanoparticles to construct a "protocell" [48]. The organic–inorganic multicomponent nanocomposites can synergistically combine the advantage of mesoporous silica nanoparticles with extraordinarily high drug loading capacity, and liposome with enhanced lateral bilayer fluidity. Figure 18.5a, b shows the selective

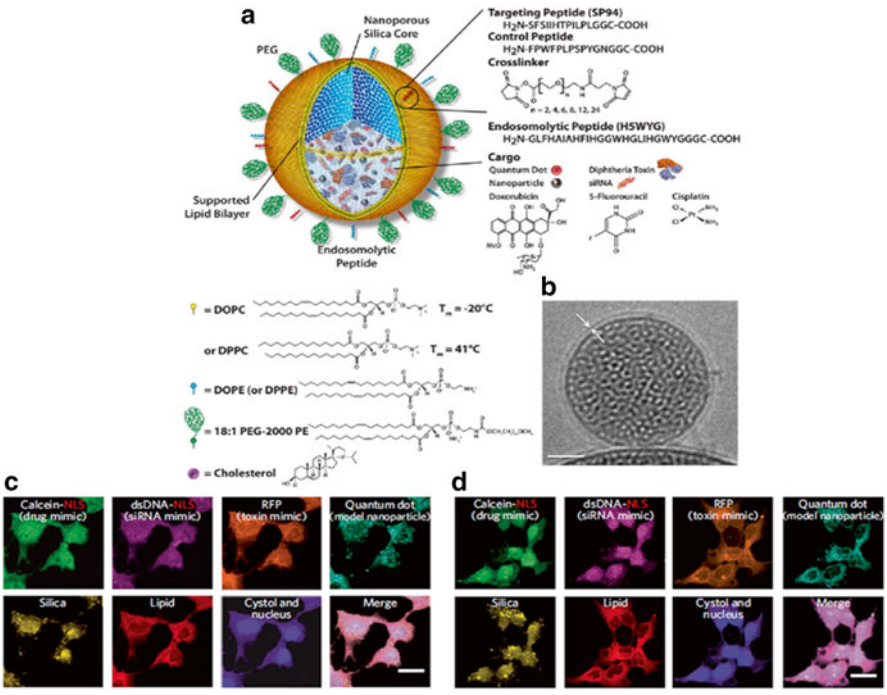

Fig. 18.5 (**a**) Schematic representation of a protocell and (**b**) cryo-TEM image of a protocell. Confocal imaging of targeted delivery of multicomponent cargos in protocells to Hep3B cells for 4 h (**c**) and 12 h (**d**) at 37 °C. Alexa Fluor 532-labeled nanoporous silica cores (*yellow*) loaded with calcein (*green*), Alexa Fluor 647-labeled dsDNA oligonucleotide (*magenta*), red fluorescent protein (*orange*), and CdSe/ZnS Quantum Dots (*teal*). Cargos were sealed in the cores by fusion of Texas Red-labeled 1,2-dioleoyl-sn-glycero-3-phosphocholine liposomes (*red*). Scale bars are 20 μm. Reproduced from [48, 197]

and non-selective binding characteristics of peptide (SP94)-targeted protocells. These advanced nanostructures enable targeted delivery and controlled release of high concentrations of multicomponent cargos within the cytosol of cancer cells. The hybrid nanocomposites were used to deliver drugs and drug cocktails, siRNA cocktails and protein toxins, reducing the multidrug resistant. The great advantages of mesoporous silica are the high surface area and the controllable chemistry that allow for both simple and combination of diverse classes of cargos that can be delivered by endocytosis or macropinocytosis to the cells. A multicomponent mesoporous silica for drug delivery and targeting system using protocells has been shown to be taken up by the cells by a receptor-mediated endocytotic pathway, as indicated by the co-localization of the cargo, mesoporous silica, and lipid after 15 min (Fig. 18.5c) [48]. After 12 h incubation with the cells, the multifunctional delivery system released the cargo triggered by the change in pH, leading to endosomal disruption and release of the cargo into the cytosol.

In addition, mesoporous silica nanoparticles functionalized with folate and loaded with the anticancer drug camptothecin have been shown to increase the cytotoxicity in pancreatic PANC-1 cancer cells (60 % cell death based) [79]. Doxorubicin-loaded mesoporous silica surface functionalized with aptamer incubated for 24 h with cancerous HeLa cells and healthy QGY7703 cells was shown to kill about 60 % and 40 % of the cells [80], respectively. Furthermore, Rosenholm and coworkers conjugated the anticancer drug methotrexate onto the surface of mesoporous silica nanoparticles, which acted as both targeting ligand and a cytostatic drug [81]. The loaded particles induced an apoptotic cell death of about 33 % after 72 h incubation with HeLa cells, whereas no apoptosis was observed in non-cancerous HEK293 cells.

Recently, advanced mesoporous silica systems have also been developed to deliver concentrated payloads of drugs to cancerous tissues by controlling the release of the drug precisely upon activation by an external stimulus. In this case the drugs can be stored inside the pores of the particles for longer times without premature diffusional loss of the payload. Zink and coworkers developed a series of nanoswitches, nanovalves, and nanomachines based on the capping of the pores of the mesoporous silica nanoparticles by using of non-covalent chemistry and binding of caps through hydrogen bonding, donor–acceptor, and ion–dipole interactions [36]. These nanosystems have been successfully tested in vitro and in vivo for their efficiency in controlled drug delivery as well as cancer therapy.

For more detailed examples on the use of mesoporous silica nanoparticles for biomedical applications, the readers are directed to the following excellent reviews on the topic [33–40, 78].

18.2.2.2 Mesoporous Silicon

Similarly to mesoporous silica materials, also mesoporous silicon materials have been exploited as a potential platform for controlled drug delivery, imaging, and cancer therapy [7, 8, 52, 57–59, 61–63, 71, 82–85]. Most of the studies described in

the literature using mesoporous silicon materials use microparticles. For example, Ferrari and coworkers have reported in several studies the ability of mesoporous silicon microparticles to circulate in the blood stream and find tumors in vivo. The size and shape effects in the biodistribution of mesoporous silicon submicron particles after intravenous injection have been demonstrated [54, 86]. It has been shown that discoidal mesoporous silicon particles were found in most of the organs, whereas cylindrical particles were mainly cleared in liver. Remarkably, hemispherical mesoporous particles showed the highest uptake in tumors compared to other formulations. Although these particles are relatively large to infiltrate into the tumor tissues via the EPR effect, van de Ven and coworkers have observed a tumoritropic accumulation of plateloid mesoporous silicon particles (1,000×400 nm) in melanoma-bearing mice as a result of the effective adherence to the endothelial cells of the tumor vessel [87, 88].

Professor Ferrari's group has also developed a multistage targeted system that preferentially binds to the vessel wall of tumors [53, 70, 89–91]. In this system, the mesoporous silicon microparticles are loaded with imaging contrast agents/nanoparticles in order to first attach on the endothelial cells of tumor and sequentially release the diagnostic nanoparticles into the cells in a time-controlled manner, which is controlled by the biodegradation of the mesoporous silicon matrix. The decoration of the multi-stage nanovectors with biorecognition molecules, such as Ly6C antibodies, have been shown to specifically bind to Ly6C, mouse homolog of CD59, overexpressed in both endothelial cells and infiltrated macrophages of pancreatic cancer [92]. The surface modification of the mesoporous silicon for dual targeting improved their in vivo targeting efficacy up to ~20-fold in the pancreatic tumors. Similarly, E-Selectin-targeted mesoporous silicon particles have been shown to specifically bind to the bone marrow, the main organ for the bone metastases [93].

Circulating leukocytes have been known to sense their environments, transmigrate through endothelial cells, and infiltrate into sites of disease. In order to improve the blood circulation and tumor targeting efficacy, the aforementioned multistage delivery systems were decorated with biomimetic leukocyte membranes [94]. This leukocyte-membrane coating of mesoporous silicon particles was able to improve their half-life in blood circulation by reducing the clearance by the immune system, interacting with endothelial cells of tumor vessel, and finally travelling into the deep tumor tissues.

Although the aforementioned delivery systems have been demonstrated to target and deliver their cargos, they are mainly based on microparticle formulations, which is a limitation for some targeting purposes. In this regard, and although the number of studies is still limited, porous silicon nanoparticles with sizes in the range of 10–100 nm have also been investigated for in vivo tumor targeting. These nanoparticles can prolong the residence time in the blood stream by avoiding rapid renal filtration and RES uptake and accumulate in the cancerous tissues by the EPR effect. For example, Park and coworkers have developed dextran-coated mesoporous silicon nanoparticles for in vivo tumor targeting [51]. Passive tumor accumulation of the nanoparticles was observed in mice bearing human breast tumors after an intravenous injection.

In order to localize the nanoparticles into the tumors more efficiently, their surface is usually coated with multiple ligands that preferentially recognize tumor cells. As a result, multiple copies of the targeting ligands displayed on the particle's surface strongly interact with the receptors on the tumor cells, thus enhancing tumor targeting efficiency of the particles significantly, the so-called "multivalent effect." The surface modification of mesoporous silicon nanoparticles with targeting peptides (pA20-6) screened with a phage display technique was found to specifically recognize antigen receptors expressed on the surface of murine B lymphoma A20 cells [95]. These targeted mesoporous silicon nanoparticles were capable of multivalent binding to target molecules, which allowed efficient in vivo targeting without considerable toxicity. In another recent study, Rytkönen and coworkers have also developed PEGylated mesoporous silicon nanoparticles conjugated with antibodies against glutathione S-transferase for targeting applications [96]. A dual coating with PEG and targeting ligand on the nanoparticle reduced the amount of proteins nonspecifically bound to the nanoparticle surface, presumably improving the overall targeting efficiency.

In addition to the abovementioned studies, targeted delivery of small RNA interference (siRNA) therapy is also very challenging, but it has an exceptional potential due to the versatile application to silence genes. Tanaka and coworkers developed a mesoporous silicon-based delivery system composed of two carriers [90]: the first-stage carrier was composed of mesoporous microscale biodegradable mesoporous silicon microparticles [89], which allowed the loading and release of a second-stage nanocarriers composed of dioleoyl phosphatidylcholine (DOPC) nanoliposomal siRNA (siRNA-DOPC) in a sustained manner. In order to test the efficiency of the system, two orthotopic mouse models of human ovarian cancer were treated either twice weekly with siRNA-DOPC (a total dose of 30 μg EphA2-siRNA) over 3 weeks, or by a single injection with S1MP-EphA2-siRNA-DOPC (15 μg EphA2-siRNA). Importantly, such delivery system designed against the oncogene EphA2 resulted in substantial reduction of the tumor weight and burden when compared to controls. The combinatorial multistage delivery system significantly improved the pharmacokinetics of the DOPC liposome and resulted in 3-week-long sustained gene silencing of EphA2 in the tumor with substantial decrease of angiogenesis and cellular proliferation without evidence of production of ascites.

Despite the promising results of targeted mesoporous nanomaterials, these nanosystems still encounter several challenges. For example, immunotherapy is an alternative strategy to retrieve harmful unwelcome results through intentional activation of the body's own immune system to fight against cancer. In this respect, despite low numbers of experimental studies owing to the restricted understanding of the interactions between the nanomaterials and immune system, Gu and coworkers used engineered mesoporous silicon-based nanoparticles (LPSiNPs) to activate antigen presenting cells in order to alter the potency of immunomodulators [97]. FGK45 immunomodulators (an agonist antibody of CD40) can bind to antigen presenting cell receptors of CD40 to improve the activation of B-cells; thus, a 30–40-fold increase in the cellular response to the nanoparticle-based stimulators compared to free FGK45 was observed, when FGK-LPSiNPs where readily taken up by antigen presenting cells (Fig. 18.6a). Figure 18.6b shows limited presence of

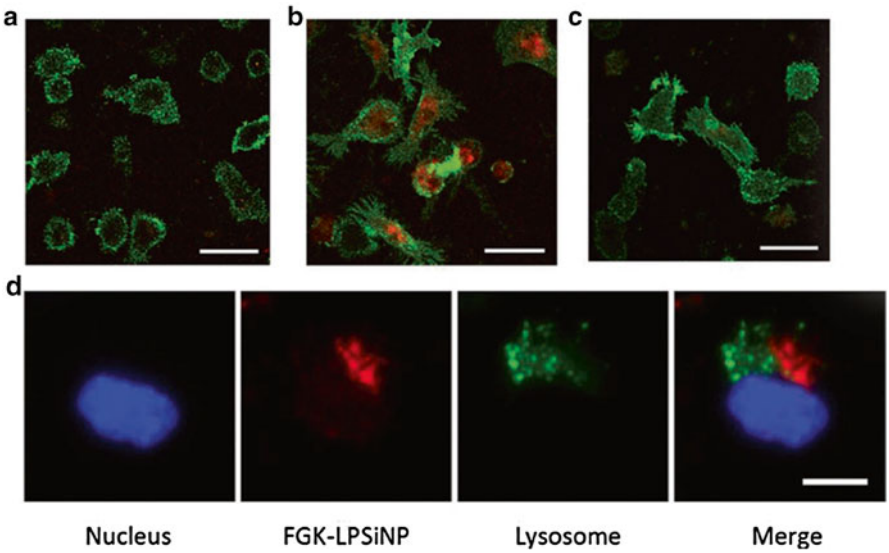

Fig. 18.6 Fluorescence microscope images of mouse bone marrow-derived dendritic cells treated with (**a**) LPSiNPs or (**b**) FGK-LPSiNPs for 1.5 h at 37 °C. (**c**) 30 min pretreatment of the bone marrow-derived dendritic cells (*green*) with free FGK45 inhibits uptake of FGK-LPSiNPs incubated with the cells for 1.5 h at 37 °C. FGK-LPSiNPs were detected by their intrinsic visible/near-infrared photoluminescence (*red*, λex = 405 nm and λem = 700 nm). The scale bars are 40 μm. (**d**) Distribution of FGK-LPSiNPs bone marrow-derived dendritic cells after 1.5 h incubation at 37 °C. The lysosomes is stained in *green* (LysoTracker; Invitrogen), the nucleus in *blue* and FGK-LPSiNPs in *red*. The scale bar is 10 μm. Reproduced from [97]

bare LPSiNPs in the mouse bone marrow-derived dendritic cells, while the FGK loaded counterparts exhibited much higher uptake of nanoparticles under the same conditions (Fig. 18.6c). To evaluate how FGK45 binding improved the internalization and induced endocytosis of FGK-LPSiNPs, cells were pretreated with free FGK45 for 30 min before incubation with FGK-LPSiNPs for 1.5 h at 37 °C, resulting in substantial block for the nanoparticles internalization (Fig. 18.6d). Overall, these results suggested the feasibility of utilizing mesoporous silicon nanoparticles for a specific tumor targeting ligand to remarkably enhance the tumor targeting efficiency of such nanosystems.

18.3 Inorganic Metal Nanoparticles: Gold and Silver

18.3.1 Gold Nanoparticles

Gold (Au) is a chemical element and metal that has been used since antiquity for both aesthetic and therapeutic purposes. The colloidal dispersions of Au (Au nanoparticles) are one of the most stable metal nanoparticles which have been

found to display remarkable properties, such as size-related electronics, optical and magnetic properties (quantum-size effects), as well as multiple applications in catalysis and biology [98].

Soluble Au was used since Ancient Greece through the Middle Ages as possessing fabulous curative powers for various diseases, such as heart and venereal problems, dysentery, epilepsy, and tumors, and for the diagnosis of syphilis [98]. Nonetheless, it was not until 1890 that the German bacteriologist Robert Koch reported the first use of gold in modern medicine, after he discovered that low concentrations of potassium Au cyanide, $K[Au(CN)_2]$, had antibacterial properties against the tuberculosis bacillus [99]. Another condition that was also reported to be successfully treated with Au was rheumatoid arthritis [100]. In addition to their therapeutic properties, the scattering cross-section of Au nanoparticles is also extremely high compared with polymeric spheres of the same size, especially in the red region of the spectrum. This property enables the Au nanoparticles to be suitable contrast agents for optical imaging in living organisms, because the light penetration depth in tissue dramatically increases with the increasing wavelength. The ability of the Au nanoparticles to bind to a wide range of organic molecules, their low level of toxicity and their strong and tunable optical absorption properties quickly establish them as suitable candidates for drug delivery applications.

18.3.1.1 Synthesis

The most common method for synthesizing Au nanoparticles is chemical reduction. This process is conducted by reducing the Au salts in the presence of reducing agents [101]. In 1857 Faraday conducted the first phase synthesis of Au colloids by reducing Au2Cl6 with phosphorous in an aqueous medium [102]. A subsequent method, which has become one of the most widely used, was the reduction of Au(III) derivatives using citrate reduction of HAuCl4 in water leading to the nucleation of Au ions to spherical nanoparticles of ca. 20 nm, which was introduced by Turkevich in 1951 [103]. This method is still used nowadays and is employed to subsequently replace the citrate ligand of the Au nanoparticles by appropriate ligands of biological interest.

Further modifications of the Turkevich method have allowed better size distribution and size control of the Au nanoparticles within the 9–120 nm range [104]. In addition, different shapes of Au nanoparticles can also be obtained by using appropriate synthesis techniques, which can be further functionalized with different targeting moieties for biological applications (Fig. 18.7).

Au nanorods with controlled aspect ratio such as nanostars, nanocages, hollow nanospheres, nanocubes, nanocrystals, and obtuse triangular bipyramids can be synthesized in a controlled fashion [105]. The stabilization of Au nanoparticles using alkenethiols was first disclosed by Giersig and Mulvaney and relied on the strong Au–S bond between the soft Au acid and the soft thiolate base [106]. Another method using the same sulphur coordination for stabilizing Au nanoparticles is the Shiffrin–Brust biphasic synthesis. This method uses HAuCl4, a thiol, tetraoctylammonium

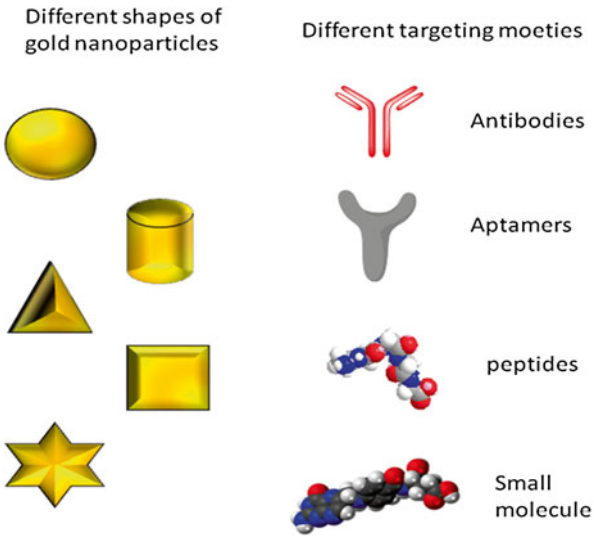

Fig. 18.7 Synthesis of different shapes of Au nanoparticles (e.g., nanorods, nanostars, nanocages, nanospheres, nanocubes, and *triangular*-shaped bipyramids) and the different targeting moieties (e.g., antibodies, aptamers, peptides, and small molecules) that can be used to functionalize the Au nanoparticles

bromide, and NaBH4 in water–toluene to yield thiolate-Au nanoparticles [107]. This allows a more trivial synthesis of thermally stable and air-stable Au nanoparticles with low dispersity and anisotropic control. The Shiffrin–Brust biphasic synthesis enables functional thiolates to be introduced, or subsequent bimolecular substitution of a thiolate ligand by such a functional thiol [108]. This allows peptides, oligonucleotides, and polymers, such as PEG to be easily attached to the Au nanoparticles' surface.

Since the solubility of these Au nanoparticles is controlled by the solubilizing properties of the terminal group of the thiolate ligands, Au nanoparticles can be transferred from an aqueous phase to an organic phase or vice versa by appropriate ligand exchange [109] Furthermore, water-soluble Au nanoparticles can also be synthesized to possess terminal carboxylate groups at the edge. The carboxyl group can therefore be used to attach amino groups from biomolecules using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl [110].

18.3.1.2 Targeting

In order to increase the therapeutic efficiency, some drugs require an appropriate carrier to overcome some of the physiological barriers encountered on its way to the intended site of action. As discussed previously, the drugs can be delivered to cells through carriers by either passive or active targeting mechanisms. By controlling the size and appending PEG molecules to the surface of Au nanoparticles, it is possible to increase the tumor accumulation of these structures through the EPR effect [111]. Other polymers such as PEI can also be covalently attached to Au nanoparticles to increase the delivery of plasmid DNA to cells due to their polycationic nature [112].

Active targeting, on the other hand, relies on the conjugation of a targeting moiety (e.g., peptide, antibody or other biological moieties) at the surface of the carrier to selectively bind to the overexpressed receptors at the cell surface. Due to their rapid cellular division and stringent demand for iron (e.g., for heme synthesis), many cancer cell types have abundant expression of transferrin receptors (TfRs). By decorating Au nanoparticles with human holo-transferrin, the intracellular delivery of these carriers is significantly increased compared with their non-targeted counterparts [113]. Figure 18.8 shows the in vivo tumor tissue and intracellular distribution of Au nanoparticles in mice bearing Neuro2A tumors after i.v. administration.

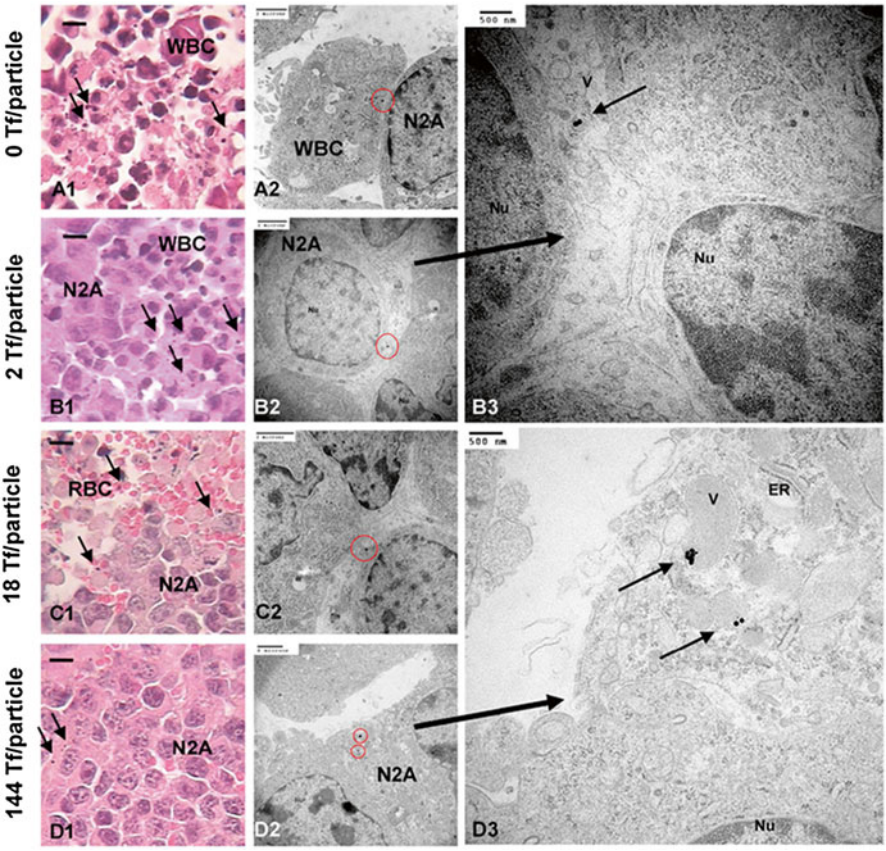

Fig. 18.8 In vivo tumor distribution of Tf-PEG-Au nanoparticles. (A–D1) Micrographs of the tumor sections. Arrows indicate Au nanoparticles (scale bar = 10 μm). Electron micrographs show particles either engulfed by leukocytes (A2) or tangentially touching Neuro2A cells (B–C2; enlarged image, B3). (D2 and enlarged image, D3) Particles with a high Tf-content (V: 144 Tf/particle) can enter Neuro2A cells. *ER* endoplasmic reticulum, *M* mitochondrion, *N2A* Neuro2A cell, *Nu* nucleus, *RBC* red blood cell, *V* vesicle, *WBC* leukocyte. Scale bars 2 μm (A1–D2) and 500 nm (B3 and D3). Reproduced from [113]

Histological examination revealed that Au nanoparticles were predominantly localized in the vicinity of leukocytes for all Tf-contents (Fig. 18.8A–B1). Intracellular accumulation of the nanoparticles inside Neuro2A cells was not observed at low Tf-contents (II–IV) (Fig. 18.8B–C1), but an increase in the Tf- content to 144 Tf/particle led to considerable nanoparticle uptake by Neuro2A cells (Fig. 8D1). A clear Tf content-dependency and intracellular nanoparticle localization was observed, but whereas II–IV localized mostly inside leukocytes (Fig. 8A2) or on the surface of Neuro2A cells (Fig. 18.8B–C2), clusters of V resided within the large endocytic vesicles of Neuro2A cells (Fig. 8D2), but not inside other leukocytes, endothelial cells, or other non-cancer cells (Fig. 8D3). Thus, the ligand contents seem not to alter the extent of bulk nanoparticle content in the tumor and, instead, the tissue and cellular distribution are favored by increasing the targeting ligand content [113].

In addition, Au nanoparticles covalently conjugated with PEG and the monoclonal antibody Herceptin, which enables recognition of breast cancer cells expressing specific tumor associated antigens, was shown to be stable and active in vitro in the presence of blood and in vivo in nude mice model for breast cancer cells [114]. Cervical epithelial cancer cells that overexpress the transmembrane glycoprotein, epithelial growth factor receptor (EGFR), were also successfully imaged by immunotargeted AuNPs [115]. In order to take advantage of the optical and electronic properties of Au nanoparticles for biological imaging, anti-EGFR antibodies conjugated to Au nanoparticles were targeted to two malignant epithelial cell lines and selected for optimal intense surface plasmon scattering using a white-light source from a conventional microscope resulting in a colored Au nanoparticle image with dark background [116]. Aptamer-conjugated Au nanoparticles were also shown to be successfully assembled on a cell membrane surface for spectral changes, providing a direct visualization of cancer cells [117].

Overall, Au nanoparticles encompass a large family of nanostructures which possess multiple attributes that makes them promising tools for biotechnology. Their unique optical properties that can be exploited for both imaging and therapeutic applications, thermal conductivity, biocompatibility and ease of stabilization and surface modification makes them prime candidates for drug delivery applications. As multifunctionality is of paramount importance for future biomedical applications, Au nanoparticles are in a privileged position to tackle some of the current challenges in theranostic applications. Au nanoparticle-based delivery vectors have continuously and successfully been investigated due to their controllable release of payloads and surface monolayer modifications, and are on the forefront of multifunctional carriers in cancer applications. It is, therefore, to be expected in the near future that numerous initiatives and studies will shed more light into the interactions between the tissues and the Au nanoparticle carriers, while even more complex systems for the theranostics applications will be envisioned. Despite all the potentials, major concerns about the in vivo accumulation and degradation of these materials are still hindering their full translation into clinic.

18.3.2 Silver Nanomaterials

Silver (Ag) is a metallic chemical element that has also an extensive historical background for medical use [118]. For centuries Ag has been known to have bactericidal properties and the effect of Ag for rendering water potable was recognized dating back to 1000 B.C. [119]. In light of this empirical evidence, in the nineteenth century, it was reported that very low concentrations of Ag had a remarkable antimicrobial effect. This deleterious effect of metal ions on bacterial activity was later coined "oligodynamic effect" [120].

The biological activity of Ag has been suggested to stem from the presence of the positively charged Ag+ ion, which denatures enzymes of the target cell or organism by actively binding to the negatively charged reactive groups, inhibiting cell replication [121]. In addition to the antimicrobial effect, Ag was also a used as a remedy for tetanus, rheumatism, colds and gonorrhoea. The treatment of burn wounds was another use of Ag which persisted for centuries [122]. However, with the advent of antibiotics in the beginning of the twentieth century and several pathological conditions arising from the use of Ag, this element was progressively discontinued from clinical use [123]. There are currently two examples of silver-based compounds used in the clinic: Ag sulfadiazine [124] and Actisorb® Ag [125]. Ag nanoparticles with a size between 1 and 10 nm were found to bind to HIV-1 and drastically inhibit the HIV infection compared to a bovine serum albumin (BSA) surface-modified Ag particles, which are promising for further studies of Ag nanoparticles in HIV infection [126].

18.3.2.1 Synthesis

The synthesis of Ag nanoparticles can be achieved by chemical reduction of a Ag salt by a reducing agent such as NaBH4, citrate and ascorbate [127]. Strong reducing agents, such as borohydride, yield small monodisperse Ag nanoparticles, but controlling the synthesis to obtain larger Ag particles with this process is rather difficult. Conversely, the use of a weaker reductant, such as citrate, resulted in a slower reduction rate but increased particle polydispersion.

This limitation can be partially surmounted by using a two-step reaction process. The strong reducing agent is applied first to ensure control over particle size, followed by a weaker reducing agent to enlarge the small Ag nanoparticles. This two-step process was found to increase the Ag nanoparticles size from about 20–45 to 120–170 nm [128]. The use of reducing agents, however, is associated with environmental toxicity and biological hazard, and alternative synthesis methods for Ag colloid nanoparticles are actively sought. Another synthetic route that was found to produce size-controlled Ag nanoparticles in a one-step method was based on a Tollens process, in which the ammoniacal Ag was reduced by glucose [129]. This process enabled stable Ag nanoparticle aqueous dispersions, ranging from 50 to 200 nm, or decorative Ag coatings on microspheres to be obtained.

Wu and coworkers reported a glutathione assisted preparation method of Ag nanoparticles passivated with BSA resulting in tunable optical features that were dependent on size and surface coverage, which could be potentially relevant for biomedical applications [130].

18.3.2.2 Targeting

Although colloidal Ag has proven undeniable bactericidial effects, Ag nanoparticles have not yet been applied extensively in nanoparticle-based drug delivery applications. This has been most likely due to the difficulty of Ag nanoparticle synthesis and surface modification, reduced stability when functionalized according to popular salt-aging techniques, and concerns regarding Ag-toxicity. However, as mentioned above, the clinical use of Ag nanoparticles as effective antimicrobial agents in burns and wound care, in addition to in vivo studies which supported a positive safety profile for systemic Ag nanoparticle exposure, have prompted a renewed interest in these nanomaterials [131–133]. The use of Ag nanoparticles to abrogate vascular endothelial growth factor (VEGF) induced angiogenesis in bovine retinal endothelial cells and in vivo angiogenesis using mice and rat models, which provide excellent examples of further uses of Ag nanoparticles [134].

The weaker chemical and colloidal stability of Ag nanoparticles when compared with other noble metals makes the successful conjugation of DNA very difficult [135]. However, targeted Ag nanoparticles of sizes 60–80 nm and decorated with thiol-terminated photolabile DNA oligonucleotides were prepared and given to cells and demonstrated great potential to improve the pharmacokinetic properties and precisely controlled activity in vivo [136]. For example, conjugates of Ag and oligonucleotides based upon DNA with cyclic disulfide-anchoring groups have been prepared and displayed distance-dependent optical properties and highly cooperative binding properties, which are envisioned to convey tremendous benefits in molecular diagnostic labeling and plasmonic devices. Figure 18.9 shows the confocal microscopy results demonstrating the successful delivery and photocaged effect of Ag nanocarriers for antisense DNA oligonucleotides. The Ag nanoparticle-antisense oligonucleotides decreased the expression level of CD54 upon photoactivation [136]. Successful knockdown was observed by reduced membrane labelling of antihuman CD54 antibody when comparing non-exposed (Fig. 18.9a, images 2c, d) and exposed (Fig. 18.9a, images 3c, d and 4c, d) Ag nanoparticle-oligonucleotide treated cells. Interestingly, the Ag nanoparticle-oligonucleotides showed less nuclear entrapment than polymer-complexed oligonucleotides, with significantly increased antisense activity upon photoactivation (Fig. 18.9b).

The control over a specific number of DNA ligands at the surface of the Ag nanoparticles enables mimicry of the precise and valence-dictated molecular self-assembly behavior of these nanomaterials and is, therefore, extremely useful for obtaining DNA-programmable materials. The synthesis of Ag nanoparticles–DNA bio-nano-conjugates bearing a discrete number of DNA ligands was also reported in

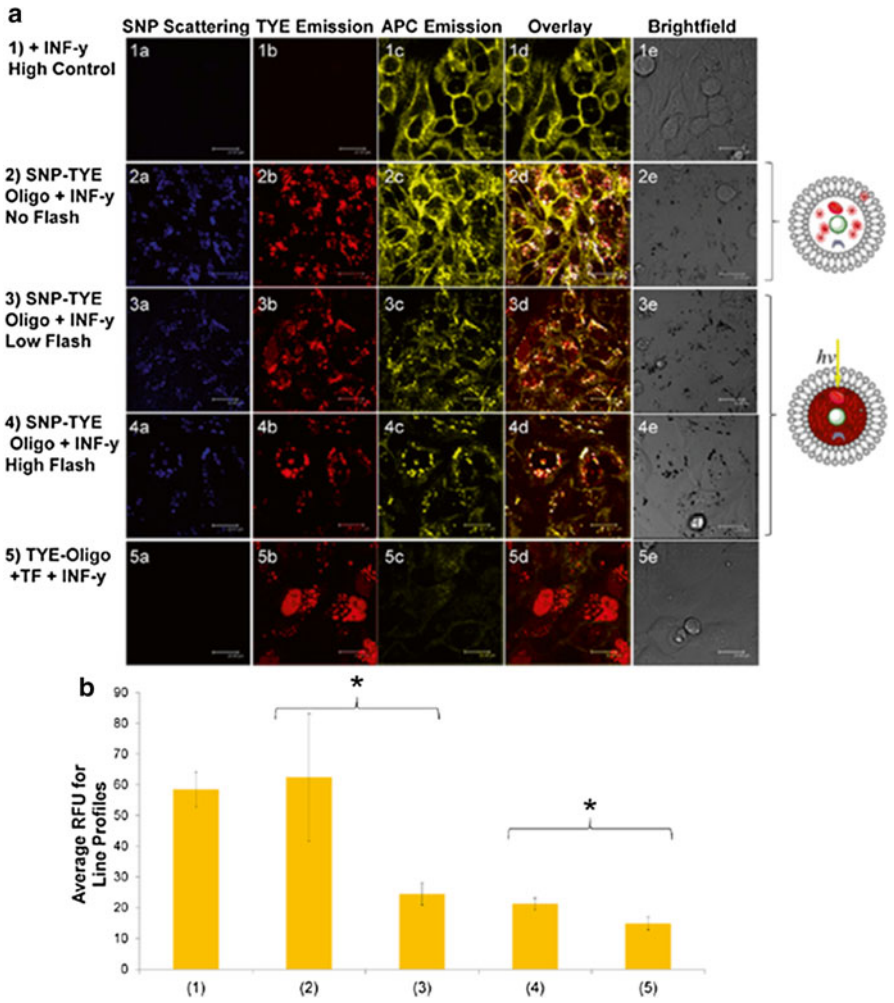

Fig. 18.9 Antisense activity of photoactivated Ag nanoparticle-tethered oligonucleotides [SNP-TYE-NPE(1n)-oligo] conjugates. (a) CD54-expressing HeLa cells (1) without antisense treatment are compared to SNP-oligo conjugate treated cells in both (2) nonactivated and (3, 4) photoactivated cases. (5) TurboFect (TF) transfected TYE-oligo treated HeLa cells are used as control. (**a**) Ag particle scattering (488/488 nm), (**b**) TYE fluorescence (549/563 nm), (**c**) APC-conjugated antihuman CD54 fluorescence (633/680), (**d**) overlay, and (**e**) bright-field graphs. Overlay regions are magenta (SNP scattering plus TYE emission) and *white* (SNP, TYE, plus APC emission). (**b**) Cell periphery fluorescence line scans of cells in representative images for APC-antibody labelling intensities. Significance was determined using Student's *t* test (*$p<0.01$; $n>3$). Reproduced from [136]

the absence of thiols or surfactants for the surface passivation of Ag nanoparticles and could therefore be helpful in maintaining Ag nanoparticle activities for surface-dependent applications, such as catalysis [137]. Surface decoration of Ag nanoparticles with other molecules, such as glucose, lactose, oligonucleotides and their

combinations, was also found to modulate cytotoxicity and, more importantly, cellular uptake in mouse and human cell models [138].

Despite the long history, the actual mechanisms of Ag nanoparticles toxicity are not yet completely elucidated, and there exists a great degree of uncertainty with regard to their actual toxicity. Still, there is a great interest in the use of this material for biomedical applications. The proven bactericidal effect, together with their great sensitivity for optical sensing, has enabled them to be used as disease markers and single particle assays. It is fair to assume that the targeting of Ag nanoparticles is still in its infancy and presents more technical challenges than other noble metals, such as Au and platinum, for effective surface modification. Nonetheless, these nanomaterials represent a versatile platform for molecular imaging and controlled localized denaturation or cleavage of proteins and nucleic acids, potentially useful for diagnostic or other therapeutic goals.

18.4 Inorganic Nanoparticles for Bioimaging: Quantum Dots (QDs) and Magnetic Nanoparticles

18.4.1 Quantum Dots (QDs)

18.4.1.1 Imaging

Quantum dots (QDs) are metallic particles with a diameter of a few nanometers. They are formed from semiconductor atoms from groups II–VI or III–V, e.g., CdS, CdSe, CdTe, ZnS, ZnSe, ZnO, GaAs, InAs, InP. The core of QDs is normally formed of a semiconductor material like CdSe, which is then covered by another semiconductor material having larger spectral band-gap, like ZnS: this structure increases the photostability and quantum yield for emission.

QDs are stabilized against aggregation with capping agents, which can be functionalized [139]. QDs have no intrinsic aqueous solubility and with functionalization, for example, the solubility properties can be modified or targeting agents can be added [140, 141]. Functionalization can be made by direct chemical coupling to an existing functional group or by non-covalent self-assembling. For example, proteins having positively charged domains interact with QDs having negatively modified surfaces. The solubility of QDs can be increased with thiol compounds (mercaptoacetic acid, alkylthiol-terminated DNA, thioalkylated oligoethyleneglycols, D-L-cysteine, PEG-terminated dihydrolipoic acid), or the QDs can be coated, for example, with amphiphilic diblock or triblock copolymers, silica, polysaccharides, polymers, phosphine, peptides/proteins or put inside phospholipid micelles, depending on the requested applications [142–149].

QDs have specific optical properties: high quantum yields, high molar extinction coefficients, good photostability, and broad absorption from UV to near IR combined with narrow and symmetric photoluminescence spectra [150–153]. Compared to organic dyes, the molar extinction coefficients of QDs are even 10–100 times higher.

These good optical properties have been utilized in biomedical imaging [145, 154]: fluorescent QDs can be utilized for cell labelling, biosensing, in vivo imaging, bimodal magnetic-luminescent imaging, antibody-mediated imaging, and diagnostics. Biocompatible QDs can be used for tumor targeting/imaging, metastatic cell tracking and lymph node mapping [140, 145, 155, 156].

In biomedical applications, the widely used but toxic CdSe-based QDs can be often replaced by more suitable InP-ZnS QDs coated with other materials, which prevents the leakage of toxic heavy metals. Yong and coworkers showed with InP-ZnS QDs the possibility for in vivo cancer detection and therapeutic applications [157]. They made imaging for live pancreatic cancer cells (Fig. 18.10). In primary and metastatic pancreatic cells the antigen receptors for anticlaudin 4 are overexpressed and, therefore, the QDs were conjugated with anticlaudin 4. Also, a polyclonal antibody, anti-PSCA, was conjugated to the QDs and no morphological damages to the cells were observed with QD handling demonstrating low cytotoxicity. All the results convinced the researchers that the InP-ZnS bioconjugates can be potential biocompatible targeted nanoprobes for diagnosing human pancreatic cancer cells.

QDs cytotoxicity is dependent on the protecting inorganic shell layers, surface charge and charge distribution [158–160]. Besides possible toxicity, other drawbacks related to the use of QDs are their higher price compared to organic dyes. Also, nonspecific binding and aggregation that can lower the fluorescence as well as the larger size compared to organic dyes may be problematic. For imaging purposes, the QDs can be formulated with different materials. For example, Schliehe and coworkers used CdSe QDs and encapsulated them inside poly(lactic-co-glycolic acid)-microspheres, which were used for antigen carrier devices in vaccination [149]. Intracellular localization of CdSe-labeled microspheres within dendritic cells or macrophages was detected with fluorescence microscopy as a function of time. In addition, QDs can also be formulated inside of hyperbranched macromolecule networks forming gels, where the fluorescence is even higher than with the corresponding solutions [161].

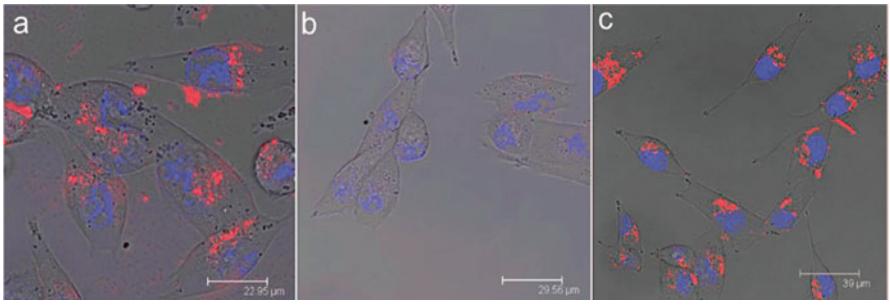

Fig. 18.10 Confocal microscopic images of (**a**) MiaPaCa cells treated with anticlaudin 4-conjugated InP/ZnS QDs; (**b**) MiaPaCa cells treated with unconjugated InP/ZnS QDs; and (**c**) MiaPaCa cells treated with anti-PSCA-conjugated InP/ZnS QDs. Reproduced from [157]

18.4.1.2 Targeting

Functionalization for targeting can be achieved, for example, by conjugating the QDs with folic acid [141]. The folic acid-InP-ZnS-QDs were shown to accumulate in the multivesicular bodies of human nasopharyngeal epidermal carcinoma cells. The delivery was confirmed to be due to the presence of folate receptors in the cells, which property can be utilized also in deep tissue imaging. QDs can also be utilized to study the cellular level phenomena. The intercellular transport of internalized CdTe QDs through tunneling-nanotubes between human hepatocellular carcinoma cells showed that the intercellular transportation was mediated by active processes [162]. Also, QDs transportation within lysosomes was noticed.

Inorganic nanoparticles like QDs and Ag and Au nanoparticles are efficient for skin penetration and permeation studies [163–167]. The importance of the nano-vehicle's shape and surface charge have been demonstrated, particularly the effect of the different surface charges (negative, positive and neutral) and shape (spherical and ellipsoidal) on skin penetration [168]. The results showed penetration of spherical particles into the epidermis and dermis independently of the surface charge of the particles. Cationic and neutral ellipsoidal QDs behaved similarly, while anionic ellipsoidal QDs penetrated the skin very slowly. Upadhyay and coworkers conjugated diphtheria toxoid vaccine into QDs and administered them transdermally to mice [92]. This transdermal delivery route induced as strong immune response through the intramuscular injection of the vaccine.

Besides the surface properties and functional groups, also the particle size of the nano-vehicles utilizing QDs can affect the biodistribution. As already mentioned, the size of QDs is typically a few nanometers, but when they are combined with other materials/other structures (e.g., polymeric particles, micelles, dendrimers), the sizes of the formed nano-vehicles are typically from some tens of nanometers to some hundreds of nanometers. Popovic and coworkers studied the effect of particle size of water-soluble QDs on the in vivo distribution [169]. They noticed that smaller (60 nm) QDs were able to extravasate and diffuse away from blood vessels, while larger (125 nm) QDs were hindered by interstitial transport barriers.

In biomedical applications the drug delivery is often combined to imaging purposes, also with QD applications. Intravenously administered ZnS-capped CdSe QDs functionalized with peptides were used for tumor targeting, imaging and drug delivery purposes in mice [140]. Two different peptides, F3 and LyP-1, were used, and depending on the coating, QDs were accumulated either at blood vessels (F3) or lymphatic vessels (LyP-1) in the tumors. When GFE-1 peptide was used as coating agent, QDs accumulated to lung vasculature showing possibility of QDs to target also to normal organs. Cai and coworkers showed in vivo imaging and targeting to tumor vasculature with the aid of RGD peptide-labeled CdTe-ZnS QDs, and suggested even the possibility of imaging guided surgery [170]. Figure 18.11 shows the Spectral the fluorescence signal observed for the accumulation of the modified QDs in the tumor, which produced a steady increase over time in the tumor fluorescence intensity in mice injected with QD705-RGD. Doxorubicin and RNA aptamer have been successfully combined to QDs for targeted drug delivery and imaging purposes

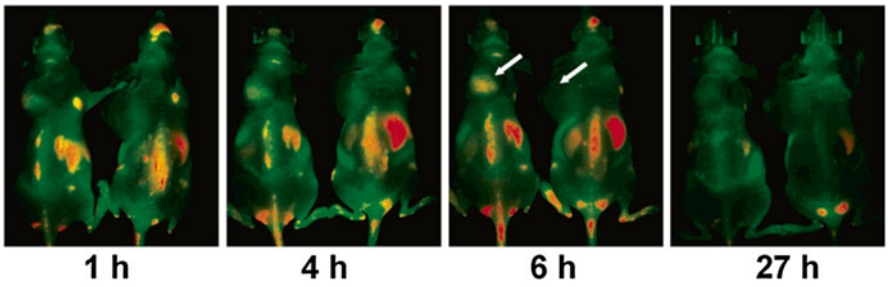

Fig. 18.11 In vivo NIR fluorescence imaging of U87MG tumor-bearing mice (left shoulder, pointed by *white arrows*) injected with 200 pmol of QD705-RGD (*left*) and QD705 (*right*), respectively. Reproduced from [170]

in prostate cancer cells [171]. The system utilized RNA aptamer ability to bind prostate-specific membrane antigen. In addition, Wang and coworkers confirmed the anti-dengue viral activity of algae extract with a QD system [172]. The nano-vehicles were formulated by encapsulating CdSe-ZnS QDs with alginate, which were further complexed with the dengue virus.

QDs can be combined with other nanomaterials for multifunctional devices [173]. For example, colloidal stable multifunctional devices were built-up by binding together QDs, magnetic particles, and antibodies using proteins (barnase and barstar) [174]. These devices interacted with cancer cells causing fluorescent labelling of the cells, and the devices were also responsive to magnetic fields. Combination of Au nanoparticles with QDs as core, covered by amphiphilic hyperbranched block polymer and loaded with camptothecin, was shown to be an efficient targeted anticancer drug delivery system [175]. Several other examples of the utilization of QDs in pharmaceutical applications are summarized in Table 18.2.

18.4.2 Magnetic Nanoparticles

The ability to manipulate and remotely control specific cellular components both in vitro and in vivo, would provide clinicians and scientists with powerful tools for investigating cell function and molecular signalling pathways, and to probe the mechanisms governing ion-channel activity, elucidating pathways that may lead to apoptosis, the production of proteins and stress responses, and to develop new treatments for medical conditions [176]. Nanomagnetic actuation provides "optical tweezers" for the nano-indentation and non-magnetic nanopatterned arrays, which have been used to control cell adhesion, integrin clustering, and intercellular signalling. The magnetic field can be coupled to magnetic nanoparticles to act within target cells or tissues. The functional outcome can then be varied dynamically by varying the strength of the applied magnetic field. The biggest advantage of magnetic applications is the precision afforded by the technique, which can be used to

Table 18.2 Examples of the utilization of QDs in pharmaceutical research

Vehicle	Purpose of the study	Reference
Camptothecin-loaded ZnO QDs Au conjugated nanoparticles	Tumor-targeted drug delivery system	[175]
ZnS-capped CdSe QDs coated with different peptides	Targeting to tumors or health lungs was dependent on the peptide coating	[140]
QDs-diphtheria toxoid vaccine	Transdermal vaccine delivery; drug penetration and efficiency study	[92]
InP-ZnS QDs	Imaging/diagnostics	[157]
Protein coated CdS QDs	Interaction studies between proteins and QD	[198]
PEG-b-polyacrylic acid coated Gd_2O_3:Er^{3+}, Yb^{3+} nanoparticles and nanorods	Imaging; biomarkers	[199]
Peptide-labeled CdTe-ZnS QDs	Drug targeting and imaging	[170]
CdSe-CdS QDs	Imaging of biomolecular processes within cells	[200]
Carboxyl-CdSe-ZnS-QD-siRNA-PEI complex	Cellular level studies: intracellular uptake and unpacking kinetics	[147]
ZnS coated Cd-Se QDs	Imaging	[144]
CdSe-ZnS-QD-aptamer-doxorubicin	Cancer targeted drug delivery; imaging; sensing	[171]
CdSe-ZnS QDs	Drug delivery and sensor applications in nanogels	[201]
Captopril-QDs	Biodistribution studies	[202]
ZnO-ZnS QDs	Drug binding studies	[203]
Hyaluronic acid-CdSe-CdS-ZnS-QD conjugates	Bioimaging	[160]
QD labeling	Intracellular dissociation of polymer–pDNA complex	[204]
CdSe-ZnS-QDs	Cellular uptake	[205]
Single-domain antibody functionalized CdTeSe-CdS QDs	Pancreatic cancer; targeting; imaging	[206]

target, manipulate, and activate, for example, stem cells [177] or even individual ion-channels or surface receptors on specific cells within a culture [178].

Superparamagnetic iron oxide nanoparticles (SPIONs) have been widely studied as a feasible nanotheranostics approach for tumor imaging and targeted anticancer drug delivery [179–182]. SPIONs are contrast agents for magnetic resonance imaging (MRI) since they induce a shorter T2 relaxation (transverse or spin-spin relaxation), producing decreased signal intensity on a T2-weighted image [183]. SPION products have been clinically used as contrast agents due to their high contrast effects and biocompatibility. The physiologically most relevant water-soluble SPIONs are composed of an iron-oxide magnetic core coated with hydrophobic oleic acid and a surface of amphiphilic polymers [184]. The surface polymers stabilize the nanoparticles and provide active functional groups for the attachment (bioconjugation) of targeting ligands. Furthermore, as presented previously with other

types of inorganic nanoparticles, the surface coating of the SPIONs with biocompatible polymers such as PEG can reduce reticuloendothelial system uptake as well as nonspecific interactions with plasma membranes. For example, it has been demonstrated that the cancer-targeting ligand labelled SPIONs could specifically bind to cancer cells and accumulate in tumor tissues [185, 186].

Among others, Jun and coworkers have developed synthetically controlled magnetic nanocrystal systems that correlate well to the nanoscale tunabilities in terms of size, magnetism, and induced nuclear spin relaxation processes [187]. SPIONs are often prepared by ligand-exchange mechanism in a simple and versatile extraction method [188]. Solutions of iron (III) and iron (II) chloride are dissolved in water and purged with nitrogen for 20 min. Under conditions of vigorous stirring, sodium hydroxide is added drop-wise until the pH reaches 8.5. The dispersion is washed with water and nitric acid by centrifugation, and the pellet is sonicated and redispersed in water. The purified uncoated SPIONs have an average hydrodynamic size of 70 nm and a high positive zeta potential of +40 mV.

The developed high-performance magnetic nanocrystal probe system has been shown suitable, for example, for the diagnosis of breast cancer cell lines. The ligand shell was conjugated with the cancer-targeting antibody Herceptin due to its specific binding properties against a HER2/neu receptor that is overexpressed in breast cancer cells [187]. Conjugation of the WSIO nanocrystals with Herceptin was performed to evaluate the cancer cell diagnosis activity of WSIO-Herceptin probe conjugates, and the binding specificity by treating them to a breast cancer cell line, SK-BR-3, which possesses overexpressed HER2/neu cancer markers. In the T2-weighted MR images, treatment of WSIO-Herceptin probe conjugates to the cell lines resulted in significant darkening of the MR images compared to non-treated cell lines, indicating selective binding of the probe conjugates to the target cancer cells. In contrast, no noticeable differences were observed between the non-treated and WSIO-irrelevant antibody control conjugate-treated cells, which further confirmed the minimal nonspecific adhesion of the conjugates and excellent selectivity.

Zou and coworkers studied the antibody- and fluorescence-labeled SPIONs as nanotheranostics for MRI and fluorescence imaging of cancer cells and pH-dependent intracellular drug release [189]. The nanoparticles (10 nm) were coated with amphiphilic polymers and PEGylated. The antibody (HuCC49ΔCH2) and fluorescent dye (5-FAM) were conjugated to the PEGylated iron oxide nanoparticles (IONPs). Anticancer drugs doxorubicin (Dox), azido-doxorubicin (Adox), MI-219, and 17-DMAG were encapsulated into the IONPs. PEGylation and conjugation with antibody and 5-FAM increased the SPION size from 18 to 44 nm. The targeting antibody HuCC49ΔCH2-SPIO increased the cancer cell targeting (both human colon cancer cell line LS174T (Fig. 18.12) and human skin cancer cell line A375) and decreased the T2 values in MRI of the LS174T cells from 117 to 56 ms. The cancer drugs Dox, MI-219 and 17-DMAG showed pH-dependent release, while Adox did not. In addition, fluorescent imaging demonstrated the accumulation of HuCC49ΔCH2-SPION nanotheranostics in endosomes/lysosomes. The encapsulated Dox was released in the acidic lysosomes and diffused further into cytosol and

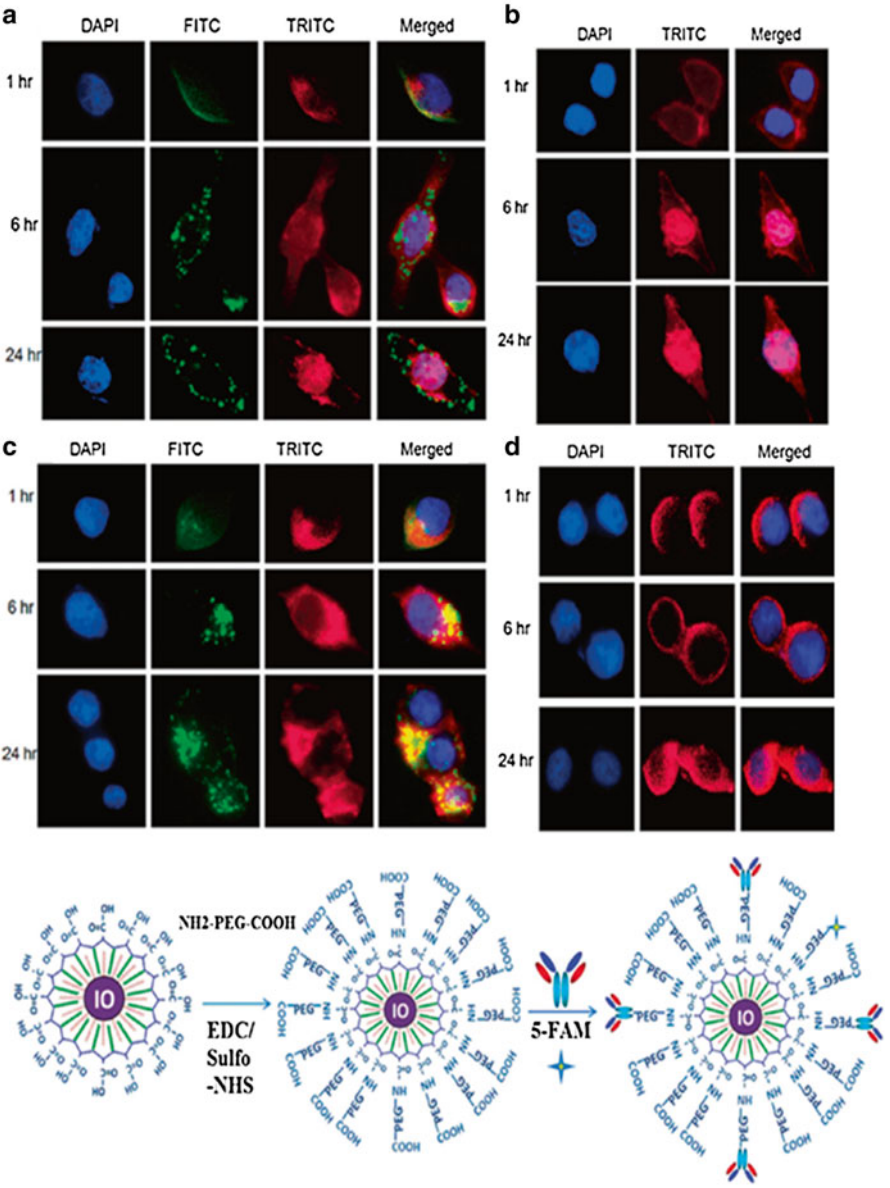

Fig. 18.12 Intracellular distribution of Dox, Adox, and HuCC49ΔCH2-SPIOs in LS174T cells. Fluorescent images of cells incubated with Dox (**a**); HuCC49ΔCH2-SPIOs loaded with Dox (**b**); Adox (**c**); and HuCC49ΔCH2-SPIOs loaded with Adox (**d**). *Green color* shows the localization of SPIONs (5-FAM). Nuclei are stained in *blue color. Red color* shows the distribution of Dox or Adox. The *yellow color* in the merged images indicates colocalization of SPIONs and Dox or Adox. (**d**) SPION PEGylation and conjugation with antibody and 5-FAM. Reproduced from [189]

nuclei, while the release of the encapsulated Adox was clearly limited. It was further shown that the HuCC49ΔCH2-SPION nanotheranostics targeted and delivered more Dox to the LS174T cells than nonspecific IgG-SPIONs (higher IC50 of 1.44 μM vs. 0.44 μM, respectively). Overall, the developed HuCC49ΔCH2-SPION nanotheranostics may be regarded as an integrated platform for cancer cell imaging, targeted anticancer drug delivery with a pH-dependent drug release.

In another work, Kumar and coworkers studied the delivery and targeting of magnetic nanoparticles that were conjugated with plasmid DNA expressing enhanced green fluorescent protein (EGFP) and then coated with chitosan [190]. The nanoparticles were injected into mice through the tail vein and directed to the heart and kidneys by means of external magnets of 25 G or 2kA–kA/m, and monitoring the elevated expression levels of EGFP in these sites. The chitosan-coated magnetic nanoparticles were also used for the coupled targeting of atrial natriuretic peptides and carcinoembryonic antigen antibodies towards human embryonic kidney 293, human A549 lung adenocarcinoma, and human lung diploid WI-38 (normal human fibroblast control) cell lines. These studies demonstrated the potential capacity of simple external magnetic field in targeting drugs to specific sites in the cells and inside the body without the need to even functionalize the iron oxide-based nanoparticles.

Liong and coworkers studied simultaneously aspects of drug delivery, magnetic resonance and fluorescence imaging, magnetic manipulation, and cell targeting by superparamagnetic iron oxide nanocrystals that were encapsulated inside multifunctional mesostructured silica spheres labelled with fluorescent dye molecules and coated with hydrophilic groups to prevent aggregation [191]. Camptothecin or paclitaxel cancer drug-loaded nanoparticles were tested on pancreatic cancer cell lines PANC-1 and BxPC3. The magnetic SPIONs alone were not toxic to the cells at the concentrations used, but the drug-loaded nanoparticles caused clear cytotoxicity in both the cell lines. Folic acid was used as the targeting component, because α-folate receptor is upregulated in several types of human cancers [192]. The effect of folic acid targeting was studied on the cellular uptake of cancer cells PANC-1 and compared to human foreskin fibroblasts (HFF) [191]. Western blot and reverse transcription polymerase chain reaction confirmed the overexpression of α-folate receptor on the PANC-1 cells at both protein and mRNA levels, but not on the HFF. Thus, the overexpression of folate receptor on PANC-1 cells facilitated recognition of the folate-modified nanoparticles and increased the particle uptake through the folate receptor-mediated endocytosis [193, 194].

According to Fan and coworkers, an ideal theranostic nanomaterial should possess several features [195], including: (1) high selective accumulation in the diseased tissue; (2) deliver selectively an effective therapeutic action; and (3) be safe and undergo biodegradation with nontoxic by-products. Their solution for an effective theranostic material development was the isolation of targeted rare tumor cells from the whole blood and imaging and photothermal destruction of the cancer cells using a theranostic magnetic core-plasmonic shell star shaped nanoparticles. Whole blood samples were spiked with SKBR-3 cancer cells and the experimental data showed that Cy3 attached S6 aptamer conjugated theranostic plasmonic/magnetic

nanoparticles could be used for fluorescence imaging and magnetic separation even at 0.001 % mixtures. A targeted photothermal 1,064 nm NIR light at 2–3 W/cm^2 for 10 min resulted in selective irreparable cellular damage to most of the SK-BR-3 cancer cells. Plasmonic Au-coating on magnetic nanoparticles was very useful for stabilizing high-magnetic-moment nanoparticles in corrosive biological conditions in the human body. Au-coating eliminated the toxicity of iron nanoparticles. The magnetic core exerted a high demand for the circulating tumor cell separation and enrichment, and the plasmonic shells were used as the photothermal material for the hyperthermic destruction of cancer cells using NIR light.

18.5 Conclusions

In this chapter, we review briefly some of the important aspects of inorganic nanoparticles in nanomedicine and describe their potential applications as nanocarriers or agents for biomedical applications, particularly for imaging/diagnostics and targeted therapy. We address some of the important aspects of the selected inorganic nanomaterials, including mesoporous silica- and silicon-based nanomaterials, Au, Ag, QDs, and other magnetic nanoparticles, in terms of their fabrication/synthesis and targeting/imaging properties relevant for biomedical applications.

Inorganic nanoparticles hold great potential in drug delivery, diagnostics, and in vivo imaging. These engineered nanomaterials can boost drug efficacy and can improve drug targeting to specific sites within the body, therefore making treatment less toxic and invasive. For example, while fluorescent QDs and magnetic nanoparticles are employed for advanced imaging, mesoporous silica- and silicon-based nanomaterials serve as nanocarrier platforms for wide variety of therapeutics and can be easily biofunctionalized for, say, cancer therapy.

Although the current research and development of several inorganic nanoparticles is very active, several issues still remain a matter of debate, such as the possible side effects, the long term effects of biodistribution and accumulation, as well as the toxicity effects that should not be overlooked. Nevertheless, the current achievements are rather encouraging and further research is required on the interaction of these inorganic nanomaterials with biological systems from the perspective of safe use and for the translation of these nanomaterials into the clinic. Despite the current advances in these areas of research, in vivo evaluation of the safety and efficacy of these inorganic nanoparticles are still in its infancy, but such studies can open up avenues for potential groundbreaking on the prevention, diagnosis, and treatment of some diseases like cancer.

Acknowledgments Dr. Hélder A. Santos acknowledges the Academy of Finland (projects numbers 252215 and 256394), the University of Helsinki and the European Research Council under the European Union's Seventh Framework Programme (FP7/2007–2013)/ERC Grant agreement number 310892 for financial support. Dr. Luis M. Bimbo acknowledges the Finnish Cultural Foundation for financial support.

References

1. Son SJ, Bai X, Lee SB (2007) Inorganic hollow nanoparticles and nanotubes in nanomedicine part 1. Drug/gene delivery applications. Drug Discov Today 12:650–656
2. Son SJ, Bai X, Lee SB (2007) Inorganic hollow nanoparticles and nanotubes in nanomedicine part 2: imaging, diagnostic, and therapeutic applications. Drug Discov Today 12:657–663
3. Sanvicens N, Marco MP (2008) Multifunctional nanoparticles–properties and prospects for their use in human medicine. Trends Biotechnol 26:425–433
4. Sekhon BS, Kamboj SR (2010) Inorganic nanomedicine–part 2. Nanomedicine 6:612–618
5. Sekhon BS, Kamboj SR (2010) Inorganic nanomedicine–part 1. Nanomedicine 6:516–522
6. Alkilany AM, Lohse SE, Murphy CJ (2012) The gold standard: gold nanoparticle libraries to understand the nano-bio interface. Acc Chem Res 46:650–661
7. Salonen J, Kaukonen AM, Hirvonen J, Lehto V-P (2008) Mesoporous silicon in drug delivery applications. J Pharm Sci 97:632–653
8. Santos HA, Bimbo LM, Lehto V-P, Airaksinen AJ, Salonen J, Hirvonen J (2011) Multifunctional porous silicon for therapeutic drug delivery and imaging. Curr Drug Discov Technol 8:228–249
9. Ferrari M (2005) Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer 5:161–171
10. Farokhzad OC, Langer R (2009) Impact of nanotechnology on drug delivery. ACS Nano 3:16–20
11. Salonen J, Laitinen L, Kaukonen AM, Tuura J, Bjorkqvist M, Heikkila T, Vaha-Heikkila K, Hirvonen J, Lehto V-P (2005) Mesoporous silicon microparticles for oral drug delivery: loading and release of five model drugs. J Control Release 108:362–374
12. Shegokar R, Muller RH (2010) Nanocrystals: Industrially feasible multifunctional formulation technology for poorly soluble actives. Int J Pharm 399:129–139
13. Muller RH, Shegokar R, Keck CM (2011) 20 years of lipid nanoparticles (sln and nlc): present state of development and industrial applications. Curr Drug Discov Technol 8:207–227
14. Muller RH, Keck CM (2012) Twenty years of drug nanocrystals: where are we, and where do we go? Eur J Pharm Biopharm 80:1–3
15. Devadasu VR, Bhardwaj V, Kumar MN (2013) Can controversial nanotechnology promise drug delivery? Chem Rev 113:1686–1735
16. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46:6387–6392
17. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2:48–58
18. Sapra P, Allen TM (2002) Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. Cancer Res 62:7190–7194
19. Xu ZP, Zeng QH, Lu GQ, Yu AB (2006) Inorganic nanoparticles as carriers for efficient cellular delivery. Chem Eng Sci 61:1027–1040
20. Sun X, Liu Z, Welsher K, Robinson JT, Goodwin A, Zaric S, Dai H (2008) Nano-graphene oxide for cellular imaging and drug delivery. Nano Res 1:203–212
21. Rosenholm JM, Sahlgren C, Linden M (2011) Multifunctional mesoporous silica nanoparticles for combined therapeutic, diagnostic and targeted action in cancer treatment. Curr Drug Targets 12:1166–1186
22. Yang X, Wang Y, Huang X, Ma Y, Huang Y, Yang R, Duan H, Chen Y (2011) Multi-functionalized graphene oxide based anticancer drug-carrier with dual-targeting function and pH-sensitivity. J Mater Chem 21:3448–3454
23. Paik T, Gordon TR, Prantner AM, Yun H, Murray CB (2013) Designing tripodal and triangular gadolinium oxide nanoplates and self-assembled nanofibrils as potential multimodal bioimaging probes. ACS Nano 7:2850–2859

24. Murphy CJ, Sau TK, Gole AM, Orendorff CJ, Gao J, Gou L, Hunyadi SE, Li T (2005) Anisotropic metal nanoparticles: synthesis, assembly, and optical applications. J Phys Chem B 109:13857–13870
25. Na HB, Song IC, Hyeon T (2009) Inorganic nanoparticles for mri contrast agents. Adv Mater 21:2133–2148
26. Lee K-S, El-Sayed MA (2006) Gold and silver nanoparticles in sensing and imaging: sensitivity of plasmon response to size, shape, and metal composition. J Phys Chem B 110: 19220–19225
27. Murphy CJ, Gole AM, Stone JW, Sisco PN, Alkilany AM, Goldsmith EC, Baxter SC (2008) Gold nanoparticles in biology: beyond toxicity to cellular imaging. Acc Chem Res 41: 1721–1730
28. Hirsch LR, Stafford RJ, Bankson JA, Sershen SR, Rivera B, Price RE, Hazle JD, Halas NJ, West JL (2003) Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance. Proc Natl Acad Sci U S A 100:13549–13554
29. Lesniak A, Salvati A, Santos-Martinez MJ, Radomski MW, Dawson KA, Aberg C (2013) Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. J Am Chem Soc 135:1438–1444
30. Canham LT (1990) Silicon quantum wire array fabrication by electrochemical and chemical dissolution of wafers. Appl Phys Lett 57:1046–1048
31. Kresge CT, Leonowicz ME, Roth WJ, Vartuli JC, Beck JS (1992) Ordered mesoporous molecular sieves synthesized by a liquid-crystal template mechanism. Nature 359:710–712
32. Lin Y-S, Tsai C-P, Huang H-Y, Kuo C-T, Hung Y, Huang D-M, Chen Y-C, Mou C-Y (2005) Well-ordered mesoporous silica nanoparticles as cell markers. Chem Mater 17:4570–4573
33. Vivero-Escoto JL, Slowing I, Trewyn BG, Lin VS (2010) Mesoporous silica nanoparticles for intracellular controlled drug delivery. Small 6:1952–1967
34. Zhao Y, Vivero-Escoto JL, Slowing I, Trewyn BG, Lin VS (2010) Capped mesoporous silica nanoparticles as stimuli-responsive controlled release systems for intracellular drug/gene delivery. Expert Opin Drug Deliv 7:1013–1029
35. Lee JE, Lee N, Kim T, Kim J, Hyeon T (2011) Multifunctional mesoporous silica nanocomposite nanoparticles for theranostic applications. Acc Chem Res 44:893–902
36. Li ZX, Barnes JC, Bosoy A, Stoddart JF, Zink JI (2012) Mesoporous silica nanoparticles in biomedical applications. Chem Soc Rev 41:2590–2605
37. Tang F, Li L, Chen D (2012) Mesoporous silica nanoparticles: synthesis, biocompatibility and drug delivery. Adv Mater 24:1504–1534
38. Vivero-Escoto JL, Huxford-Phillips RC, Lin W (2012) Silica-based nanoprobes for biomedical imaging and theranostic applications. Chem Soc Rev 41:2673–2685
39. Yanes RE, Tamanoi F (2012) Development of mesoporous silica nanomaterials as a vehicle for anticancer drug delivery. Ther Deliv 3:389–404
40. Wang K, He X, Yang X, Shi H (2013) Functionalized silica nanoparticles: a platform for fluorescence imaging at the cell and small animal levels. Res. Publication ahead of print, Acc Chem
41. Vallet-Regi M, Rámila A, Del Real RP, Pérez-Pariente J (2001) A new property of mcm-41: drug delivery system. Chem Mater 13:308–311
42. Beck JS, Vartuli JC, Roth WJ, Leonowicz ME, Kresge CT, Schmitt KD, Chu CTW, Olson DH, Sheppard EW (1992) A new family of mesoporous molecular sieves prepared with liquid crystal templates. J Am Chem Soc 1272–7863(114):10834–10843
43. Rosenholm JM, Sahlgren C, Linden M (2010) Towards multifunctional, targeted drug delivery systems using mesoporous silica nanoparticles - opportunities & challenges. Nanoscale 2:1870–1883
44. Wu S-H, Hung Y, Mou C-Y (2011) Mesoporous silica nanoparticles as nanocarriers. Chem Comm 47:9972–9985
45. Kapoor MP, Fujii W, Yanagi M, Kasama Y, Kimura T, Nanbu H, Juneja LR (2008) Environmental friendly rapid mass production synthetic process of highly ordered nanometer

sized mesoporous silica using a combination of acid–base and evaporation approach. Micropor Mesopor Mat 116:370–377
46. He Q, Cui X, Cui F, Guo L, Shi J (2009) Size-controlled synthesis of monodispersed mesoporous silica nano-spheres under a neutral condition. Micropor Mesopor Mat 117:609–616
47. Feng X, Fryxell GE, Wang L-Q, Kim AY, Liu J, Kemner KM (1997) Functionalized monolayers on ordered mesoporous supports. Science 276:923–926
48. Ashley CE, Carnes EC, Phillips GK, Padilla D, Durfee PN, Brown PA, Hanna TN, Liu J, Phillips B, Carter MB, Carroll NJ, Jiang X, Dunphy DR, Willman CL, Petsev DN, Evans DG, Parikh AN, Chackerian B, Wharton W, Peabody DS, Brinker CJ (2011) The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers. Nat Mater 10:389–397
49. Korotcenkov G, Cho BK (2010) Silicon porosification: state of the art. Crit Rev Solid State Mater Sci 35:153–260
50. Salonen J, Lehto V-P, Björkqvist M, Laine E, Niinistö L (2000) Studies of thermally-carbonized porous silicon surfaces. Phys Status Solidi A 182:123–126
51. Park J-H, Gu L, Maltzahn GV, Ruoslahti E, Bhatia SN, Sailor MJ (2009) Biodegradable luminescent porous silicon nanoparticles for in vivo applications. Nat Mater 8:331–336
52. Bimbo LM, Sarparanta M, Santos HA, Airaksinen AJ, Makila E, Laaksonen T, Peltonen L, Lehto V-P, Hirvonen J, Salonen J (2010) Biocompatibility of thermally hydrocarbonized porous silicon nanoparticles and their biodistribution in rats. ACS Nano 4:3023–3032
53. Serda RE, Godin B, Blanco E, Chiappini C, Ferrari M (2011) Multi-stage delivery nanoparticle systems for therapeutic applications. Biochim Biophys Acta 1810:317–329
54. Godin B, Chiappini C, Srinivasan S, Alexander JF, Yokoi K, Ferrari M, Decuzzi P, Liu XW (2012) Discoidal porous silicon particles: fabrication and biodistribution in breast cancer bearing mice. Adv Funct Mater 22:4225–4235
55. Salonen J, Björkqvist M, Laine E, Niinistö L (2004) Stabilization of porous silicon surface by thermal decomposition of acetylene. Appl Surf Sci 225:389–394
56. Salonen J, Lehto V-P (2008) Fabrication and chemical surface modification of mesoporous silicon for biomedical applications. Chem Eng J 137:162–172
57. Sarparanta M, Makila E, Heikkila T, Salonen J, Kukk E, Lehto V-P, Santos HA, Hirvonen J, Airaksinen AJ (2011) 18f-labeled modified porous silicon particles for investigation of drug delivery carrier distribution in vivo with positron emission tomography. Mol Pharm 8:1799–1806
58. Santos HA, Riikonen J, Salonen J, Makila E, Heikkila T, Laaksonen T, Peltonen L, Lehto V-P, Hirvonen J (2010) In vitro cytotoxicity of porous silicon microparticles: effect of the particle concentration, surface chemistry and size. Eur J Pharm Biopharm 6:2721–2731
59. Bimbo LM, Makila E, Raula J, Laaksonen T, Laaksonen P, Strommer K, Kauppinen EI, Salonen J, Linder MB, Hirvonen J, Santos HA (2011) Functional hydrophobin-coating of thermally hydrocarbonized porous silicon microparticles. Biomaterials 32:9089–9099
60. Kilpelainen M, Monkare J, Vlasova MA, Riikonen J, Lehto V-P, Salonen J, Jarvinen K, Herzig KH (2011) Nanostructured porous silicon microparticles enable sustained peptide (melanotan ii) delivery. Eur J Pharm Biopharm 77:20–25
61. Sarparanta M, Bimbo LM, Rytkonen J, Makila E, Laaksonen TJ, Laaksonen P, Nyman M, Salonen J, Linder MB, Hirvonen J, Santos HA, Airaksinen AJ (2012) Intravenous delivery of hydrophobin-functionalized porous silicon nanoparticles: stability, plasma protein adsorption and biodistribution. Mol Pharm 9:654–663
62. Sarparanta MP, Bimbo LM, Makila EM, Salonen JJ, Laaksonen PH, Helariutta AMK, Linder MB, Hirvonen JT, Laaksonen TJ, Santos HA, Airaksinen AJ (2012) The mucoadhesive and gastroretentive properties of hydrophobin-coated porous silicon nanoparticle oral drug delivery systems. Biomaterials 33:3353–3362
63. Liu D, Mäkilä E, Zhang H, Herranz B, Kaasalainen M, Kinnari P, Salonen J, Hirvonen J, Santos HA (2013) Nanostructured porous silicon-solid lipid nanocomposite: towards enhanced cytocompatibility and stability, reduced cellular association, and prolonged drug release. Adv Funct Mater 23:1893–1902

64. Boukherroub R, Wojtyk JTC, Wayner DDM, Lockwood DJ (2002) Thermal hydrosilylation of undecylenic acid with porous silicon. J Electrochem Soc 149:H59–H63
65. Sciacca B, Secret E, Pace S, Gonzalez P, Geobaldo F, Quignard F, Cunin F (2011) Chitosan-functionalized porous silicon optical transducer for the detection of carboxylic acid-containing drugs in water. J Mater Chem 21:2294–2302
66. Kovalainen M, Monkare J, Makila E, Salonen J, Lehto V-P, Herzig KH, Jarvinen K (2012) Mesoporous silicon (psi) for sustained peptide delivery: effect of psi microparticle surface chemistry on peptide yy3-36 release. Pharm Res 29:837–846
67. Zhang F, Sautter K, Larsen AM, Findley DA, Davis RC, Samha H, Linford MR (2010) Chemical vapor deposition of three aminosilanes on silicon dioxide: surface characterization, stability, effects of silane concentration, and cyanine dye adsorption. Langmuir 26: 14648–14654
68. Sweetman MJ, Shearer CJ, Shapter JG, Voelcker NH (2011) Dual silane surface functionalization for the selective attachment of human neuronal cells to porous silicon. Langmuir 27:9497–9503
69. Arroyo-Hernández M, Martín-Palma RJ, Torres-Costa V, Martínez Duart JM (2006) Porous silicon optical filters for biosensing applications. J Non Cryst Solids 352:2457–2460
70. Serda RE, Mack A, Pulikkathara M, Zaske AM, Chiappini C, Fakhoury JR, Webb D, Godin B, Conyers JL, Liu XW, Bankson JA, Ferrari M (2010) Cellular association and assembly of a multistage delivery system. Small 6:1329–1340
71. Makila E, Bimbo LM, Kaasalainen M, Herranz B, Airaksinen AJ, Heinonen M, Kukk E, Hirvonen J, Santos HA, Salonen J (2012) Amine modification of thermally carbonized porous silicon with silane coupling chemistry. Langmuir 28:14045–14054
72. Xia T, Kovochich M, Liong M, Meng H, Kabehie S, George S, Zink JI, Nel AE (2009) Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of sirna and DNA constructs. ACS Nano 3:3273–3286
73. Meng H, Xue M, Xia T, Ji Z, Tarn DY, Zink JI, Nel AE (2011) Use of size and a copolymer design feature to improve the biodistribution and the enhanced permeability and retention effect of doxorubicin-loaded mesoporous silica nanoparticles in a murine xenograft tumor model. ACS Nano 5:4131–4144
74. Rosenholm JM, Meinander A, Peuhu E, Niemi R, Eriksson JE, Sahlgren C, Lindén M (2008) Targeting of porous hybrid silica nanoparticles to cancer cells. ACS Nano 3:197–206
75. Cheng SH, Lee CH, Chen MC, Souris JS, Tseng FG, Yang CS, Mou CY, Chen CT, Lo LW (2010) Tri-functionalization of mesoporous silica nanoparticles for comprehensive cancer theranostics-the trio of imaging, targeting and therapy. J Mater Chem 20:6149–6157
76. Ferris DP, Lu J, Gothard C, Yanes R, Thomas CR, Olsen J-C, Stoddart JF, Tamanoi F, Zink JI (2011) Synthesis of biomolecule-modified mesoporous silica nanoparticles for targeted hydrophobic drug delivery to cancer cells. Small 7:1816–1826
77. Porta F, Lamers GEM, Morrhayim J, Chatzopoulou A, Schaaf M, Den Dulk H, Backendorf C, Zink JI, Kros A (2013) Folic acid-modified mesoporous silica nanoparticles for cellular and nuclear targeted drug delivery. Adv Healthc Mater 2:281–286
78. Wang Z, Xu B, Zhang L, Zhang J, Ma T, Zhang J, Fu X, Tian W (2013) Folic acid-functionalized mesoporous silica nanospheres hybridized with aie luminogens for targeted cancer cell imaging. Nanoscale 5:2065–2072
79. Lu J, Liong M, Li Z, Zink JI, Tamanoi F (2010) Biocompatibility, biodistribution, and drug-delivery efficiency of mesoporous silica nanoparticles for cancer therapy in animals. Small 6:1794–1805
80. Zhu CL, Song XY, Zhou WH, Yang HH, Wen YH, Wang XR (2009) An efficient cell-targeting and intracellular controlled-release drug delivery system based on msn-pem-aptamer conjugates. J Mater Chem 19:7765–7770
81. Rosenholm JM, Peuhu E, Bate-Eya LT, Eriksson JE, Sahlgren C, Lindén M (2010) Cancer-cell-specific induction of apoptosis using mesoporous silica nanoparticles as drug-delivery vectors. Small 6:1234–1241

82. Bimbo LM, Makila E, Laaksonen T, Lehto V-P, Salonen J, Hirvonen J, Santos HA (2011) Drug permeation across intestinal epithelial cells using porous silicon nanoparticles. Biomaterials 32:2625–2633
83. Bimbo LM, Sarparanta M, Makila E, Laaksonen T, Laaksonen P, Salonen J, Linder MB, Hirvonen J, Airaksinen AJ, Santos HA (2012) Cellular interactions of surface modified nanoporous silicon particles. Nanoscale 4:3184–3192
84. Santos HA, Hirvonen J (2012) Nanostructured porous silicon materials: potential candidates for improving drug delivery. Nanomedicine 7:1281–1284
85. Vale N, Makila E, Salonen J, Gomes P, Hirvonen J, Santos HA (2012) New times, new trends for ethionamide: in vitro evaluation of drug-loaded thermally carbonized porous silicon microparticles. Eur J Pharm Biopharm 81:314–323
86. Decuzzi P, Godin B, Tanaka T, Lee SY, Chiappini C, Liu X, Ferrari M (2010) Size and shape effects in the biodistribution of intravascularly injected particles. J Control Release 141: 320–327
87. Van De Ven AL, Kim P, Haley OH, Fakhoury JR, Adriani G, Schmulen J, Moloney P, Hussain F, Ferrari M, Liu X, Yun S-H, Decuzzi P (2012) Rapid tumoritropic accumulation of systemically injected plateloid particles and their biodistribution. J Control Release 158:148–155
88. Van de Ven AL, Wu M, Lowengrub J, Mcdougall SR, Chaplain MA, Cristini V, Ferrari M, Frieboes HB (2012) Integrated intravital microscopy and mathematical modeling to optimize nanotherapeutics delivery to tumors. AIP Adv 2:011208
89. Tasciotti E, Liu X, Bhavane R, Plant K, Leonard AD, Price BK, Cheng MM-C, Decuzzi P, Tour JM, Robertson F, Ferrari M (2008) Mesoporous silicon particles as a multistage delivery system for imaging and therapeutic applications. Nat Nanotechnol 3:151–157
90. Tanaka T, Mangala LS, Vivas-Mejia PE, Nieves-Alicea R, Mann AP, Mora E, Han H-D, Shahzad MMK, Liu X, Bhavane R, Gu J, Fakhoury JR, Chiappini C, Lu C, Matsuo K, Godin B, Stone RL, Nick AM, Lopez-Berestein G, Sood AK, Ferrari M (2010) Sustained small interfering rna delivery by mesoporous silicon particles. Cancer Res 70:3687–3696
91. Godin B, Tasciotti E, Liu X, Serda RE, Ferrari M (2011) Multistage nanovectors: from concept to novel imaging contrast agents and therapeutics. Acc Chem Res 44:979–989
92. Upadhyay P (2006) Enhanced transdermal-immunization with diptheria-toxoid using local hyperthermia. Vaccine 24:5593–5598
93. Mann AP, Tanaka T, Somasunderam A, Liu X, Gorenstein DG, Ferrari M (2011) E-selectin-targeted porous silicon particle for nanoparticle delivery to the bone marrow. Adv Mater 23:H278–H282
94. Parodi A, Quattrocchi N, Van De Ven AL, Chiappini C, Evangelopoulos M, Martinez JO, Brown BS, Khaled SZ, Yazdi IK, Enzo MV, Isenhart L, Ferrari M, Tasciotti E (2013) Synthetic nanoparticles functionalized with biomimetic leukocyte membranes possess cell-like functions. Nat Nanotechnol 8:61–68
95. Angelis FD, Pujia A, Falcone C, Iaccino E, Palmieri C, Liberale C, Mecarini F, Candeloro P, Luberto L, Laurentiis AD, Das G, Scalac G, Fabrizio ED (2010) Water soluble nanoporous nanoparticle for in vivo targeted drug delivery and controlled release in b cells tumor context. Nanoscale 2:2230–2236
96. Rytkonen J, Miettinen R, Kaasalainen M, Lehto V-P, Salonen J, Narvanen A (2012) Functionalization of mesoporous silicon nanoparticles for targeting and bioimaging purposes. J Nanomater 2012:9
97. Gu L, Ruff LE, Qin Z, Corr M, Hedrick SM, Sailor MJ (2012) Multivalent porous silicon nanoparticles enhance the immune activation potency of agonistic cd40 antibody. Adv Mater 24:3981–3987
98. Daniel MC, Astruc D (2004) Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chem Rev 104:293–346
99. Koch R (1890) On bacteriological research. August Hirsch Forest, Berlin
100. Forestier J (1934) Rheumatoid arthritis and its treatment by gold salts. Lancet 224:646–648

101. Khan JA, Kudgus RA, Szabolcs A, Dutta S, Wang E, Cao S, Curran GL, Shah V, Curley S, Mukhopadhyay D, Robertson JD, Bhattacharya R, Mukherjee P (2011) Designing nanoconjugates to effectively target pancreatic cancer cells in vitro and in vivo. PLoS One 6:e20347
102. Faraday M (1857) The bakerian lecture: experimental relations of gold (and other metals) to light. Phil Trans Roy Soc Lond 147:145–181
103. Turkevich J, Stevenson PC, Hillier J (1951) A study of the nucleation and growth processes in the synthesis of colloidal gold. Discuss Faraday Soc 11:55–75
104. Kimling J, Maier M, Okenve B, Kotaidis V, Ballot H, Plech A (2006) Turkevich method for gold nanoparticle synthesis revisited. J Phys Chem B 110:15700–15707
105. Nie X, Chen C (2012) Au nanostructures: an emerging prospect in cancer theranostics. Sci China Life Sci 55:872–883
106. Giersig M, Mulvaney P (1993) Preparation of ordered colloid monolayers by electrophoretic deposition. Langmuir 9:3408–3413
107. Brust M, Walker M, Bethell D, Schiffrin DJ, Whyman R (1994) Synthesis of thiol-derivatised gold nanoparticles in a two-phase liquid-liquid system. J Chem Soc Chem Comm 801–802
108. Templeton AC, Wuelfing WP, Murray RW (1999) Monolayer-protected cluster molecules. Acc Chem Res 33:27–36
109. Boisselier E, Astruc D (2009) Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity. Chem Soc Rev 38:1759–1782
110. Li D, He Q, Cui Y, Duan L, Li J (2007) Immobilization of glucose oxidase onto gold nanoparticles with enhanced thermostability. Biochem Biophys Res Commun 355:488–493
111. Zhang G, Yang Z, Lu W, Zhang R, Huang Q, Tian M, Li L, Liang D, Li C (2009) Influence of anchoring ligands and particle size on the colloidal stability and in vivo biodistribution of polyethylene glycol-coated gold nanoparticles in tumor-xenografted mice. Biomaterials 30:1928–1936
112. Thomas M, Klibanov AM (2003) Conjugation to gold nanoparticles enhances polyethylenimine's transfer of plasmid DNA into mammalian cells. Proc Natl Acad Sci U S A 100: 9138–9143
113. Choi CHJ, Alabi CA, Webster P, Davis ME (2010) Mechanism of active targeting in solid tumors with transferrin-containing gold nanoparticles. Proc Natl Acad Sci U S A 107: 1235–1240
114. Eghtedari M, Liopo AV, Copland JA, Oraevsky AA, Motamedi M (2008) Engineering of hetero-functional gold nanorods for the in vivo molecular targeting of breast cancer cells. Nano Lett 9:287–291
115. Sokolov K, Follen M, Aaron J, Pavlova I, Malpica A, Lotan R, Richards-Kortum R (2003) Real-time vital optical imaging of precancer using anti-epidermal growth factor receptor antibodies conjugated to gold nanoparticles. Cancer Res 63:1999–2004
116. El-Sayed IH, Huang X, El-Sayed MA (2005) Surface plasmon resonance scattering and absorption of anti-egfr antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer. Nano Lett 5:829–834
117. Medley CD, Smith JE, Tang Z, Wu Y, Bamrungsap S, Tan W (2008) Gold nanoparticle-based colorimetric assay for the direct detection of cancerous cells. Anal Chem 80:1067–1072
118. Silver S, Phung LT, Silver G (2006) Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. J Ind Microbiol Biotechnol 33:627–634
119. Russell AD, Hugo WB (1994) Antimicrobial activity and action of silver. In: Ellis GP, Luscombe DK (eds) Progress in medicinal chemistry. Elsevier Science B.V, Amsterdam
120. Von Naegelli V (1893) Deut schr Schweiz Naturforsch Ges 33:174–182
121. Bhattacharyya S, Kudgus RA, Bhattacharya R, Mukherjee P (2011) Inorganic nanoparticles in cancer therapy. Pharm Res 28:237–259
122. Klasen HJ (2000) Historical review of the use of silver in the treatment of burns. I. Early uses. Burns 26:117–130
123. Mirsattari SM, Hammond RR, Sharpe MD, Leung FY, Young GB (2004) Myoclonic status epilepticus following repeated oral ingestion of colloidal silver. Neurology 62:1408–1410

124. Fox CL (1968) Silver sulfadiazine—a new topical therapy for pseudomonas in burns. Arch Surg 96:184–188
125. Furr JR, Russell AD, Turner TD, Andrews A (1994) Antibacterial activity of actisorb-plus, actisorb and silver-nitrate. J Hosp Infect 27:201–208
126. Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A, Gao X, Lara HH, Yacaman MJ (2005) Interaction of silver nanoparticles with hiv-1. J Nanobiotechnology 3:6
127. Nickel U, Zu Castell A, Pöppl K, Schneider S (2000) A silver colloid produced by reduction with hydrazine as support for highly sensitive surface-enhanced raman spectroscopy. Langmuir 16:9087–9091
128. Shirtcliffe N, Nickel U, Schneider S (1999) Reproducible preparation of silver sols with small particle size using borohydride reduction: for use as nuclei for preparation of larger particles. J Colloid Interface Sci 211:122–129
129. Yin Y, Li Z-Y, Zhong Z, Gates B, Xia Y, Venkateswaran S (2002) Synthesis and characterization of stable aqueous dispersions of silver nanoparticles through the Tollens process. J Mater Chem 12:522–527
130. Wu Q, Cao H, Luan Q, Zhang J, Wang Z, Warner JH, Watt A (2008) Biomolecule-assisted synthesis of water-soluble silver nanoparticles and their biomedical applications. Inorg Chem 47:5882–5888
131. Percival SL, Bowler P, Woods EJ (2008) Assessing the effect of an antimicrobial wound dressing on biofilms. Wound Repair Regen 16:52–57
132. Rai M, Yadav A, Gade A (2009) Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 27:76–83
133. Qureshi AT, Monroe WT, Lopez MJ, Janes ME, Dasa V, Park S, Amirsadeghi A, Hayes DJ (2011) Biocompatible/bioabsorbable silver nanocomposite coatings. J Appl Polym Sci 120:3042–3053
134. Kalishwaralal K, Barathmanikanth S, Pandian SR, Deepak V, Gurunathan S (2010) Silver nano—a trove for retinal therapies. J Control Release 145:76–90
135. Lee J-S, Lytton-Jean AKR, Hurst SJ, Mirkin CA (2007) Silver nanoparticle – oligonucleotide conjugates based on DNA with triple cyclic disulfide moieties. Nano Lett 7:2112–2115
136. Brown PK, Qureshi AT, Moll AN, Hayes DJ, Monroe WT (2013) Silver nanoscale antisense drug delivery system for photoactivated gene silencing. ACS Nano 7:2948–2959
137. Zheng Y, Li Y, Deng Z (2012) Silver nanoparticle-DNA bionanoconjugates bearing a discrete number of DNA ligands. Chem Comm 48:6160–6162
138. Sur I, Cam D, Kahraman M, Baysal A, Culha M (2010) Interaction of multi-functional silver nanoparticles with living cells. Nanotechnol 21:175104
139. Smith AM, Duan H, Mohs AM, Nie S (2008) Bioconjugated quantum dots for in vivo molecular and cellular imaging. Adv Drug Deliv Rev 60:1226–1240
140. Akerman ME, Chan WC, Laakkonen P, Bhatia SN, Ruoslahti E (2002) Nanocrystal targeting in vivo. Proc Natl Acad Sci U S A 99:12617–12621
141. Bharali DJ, Lucey DW, Jayakumar H, Pudavar HE, Prasad PN (2005) Folate-receptor-mediated delivery of inp quantum dots for bioimaging using confocal and two-photon microscopy. J Am Chem Soc 127:11364–11371
142. Chan WC, Nie S (1998) Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 281:2016–2018
143. Mattoussi H, Mauro JM, Goldman ER, Anderson GP, Sundar VC, Mikulec FV, Bawendi MG (2000) Self-assembly of cdse – zns quantum dot bioconjugates using an engineered recombinant protein. J Am Chem Soc 122:12142–12150
144. Dubertret B, Skourides P, Norris DJ, Noireaux V, Brivanlou AH, Libchaber A (2002) In vivo imaging of quantum dots encapsulated in phospholipid micelles. Science 298:1759–1762
145. Zhou M, Ghosh I (2007) Quantum dots and peptides: a bright future together. Biopolymers 88:325–339
146. Dif A, Boulmedais F, Pinot M, Roullier V, Baudy-Floc'h M, Coquelle FM, Clarke S, Neveu P, Vignaux F, Le Borgne R, Dahan M, Gueroui Z, Marchi-Artzner V (2009) Small and stable peptidic pegylated quantum dots to target polyhistidine-tagged proteins with controlled stoichiometry. J Am Chem Soc 131:14738–14746

147. Lee H, Kim IK, Park TG (2010) Intracellular trafficking and unpacking of sirna/quantum dot-pei complexes modified with and without cell penetrating peptide: confocal and flow cytometric fret analysis. Bioconjug Chem 21:289–295
148. Guo Y, Harirchian-Saei S, Izumi CM, Moffitt MG (2011) Block copolymer mimetic self-assembly of inorganic nanoparticles. ACS Nano 5:3309–3318
149. Schliehe C, Thiry M, Tromsdorf UI, Hentschel J, Weller H, Groettrup M (2011) Microencapsulation of inorganic nanocrystals into plga microsphere vaccines enables their intracellular localization in dendritic cells by electron and fluorescence microscopy. J Control Release 151:278–285
150. Gao X, Cui Y, Levenson RM, Chung LW, Nie S (2004) In vivo cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 22:969–976
151. Medintz IL, Uyeda HT, Goldman ER, Mattoussi H (2005) Quantum dot bioconjugates for imaging, labelling and sensing. Nat Mater 4:435–446
152. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307:538–544
153. Irrera A, Artoni P, Iacona F, Pecora EF, Franzo G, Galli M, Fazio B, Boninelli S, Priolo F (2012) Quantum confinement and electroluminescence in ultrathin silicon nanowires fabricated by a maskless etching technique. Nanotechnology 23:075204
154. Mazumder S, Dey R, Mitra MK, Mukherjee S, Das GC (2009) Review: biofunctionalized quantum dots in biology and medicine. J Nanomater 2009
155. Pöselt E, Fischer S, Foerster S, Weller H (2009) Highly stable biocompatible inorganic nanoparticles by self-assembly of triblock-copolymer ligands. Langmuir 25:13906–13913
156. Walling M, Novak J, Shepard JRE (2009) Quantum dots for live cell and in vivo imaging. Int J Mol Sci 10:441–491
157. Yong K-T, Ding H, Roy I, Law W-C, Bergey EJ, Maitra A, Prasad PN (2009) Imaging pancreatic cancer using bioconjugated inp quantum dots. ACS Nano 3:502–510
158. Derfus AM, Chan WCW, Bhatia SN (2003) Probing the cytotoxicity of semiconductor quantum dots. Nano Lett 4:11–18
159. Hoshino A, Fujioka K, Oku T, Suga M, Sasaki YF, Ohta T, Yasuhara M, Suzuki K, Yamamoto K (2004) Physicochemical properties and cellular toxicity of nanocrystal quantum dots depend on their surface modification. Nano Lett 4:2163–2169
160. Bhang SH, Won N, Lee TJ, Jin H, Nam J, Park J, Chung H, Park HS, Sung YE, Hahn SK, Kim BS, Kim S (2009) Hyaluronic acid-quantum dot conjugates for in vivo lymphatic vessel imaging. ACS Nano 3:1389–1398
161. Yan J-J, Wang H, Zhou Q-H, You Y-Z (2011) Reversible and multisensitive quantum dot gels. Macromol 44:4306–4312
162. Mi L, Xiong R, Zhang Y, Yang W, Chen J, Wang P (2011) Microscopic observation of the intercellular transport of cdte quantum dot aggregates through tunneling-nanotubes. J Biomater Nanobiotechnol 2:172–179
163. Paliwal S, Menon GK, Mitragotri S (2006) Low-frequency sonophoresis: ultrastructural basis for stratum corneum permeability assessed using quantum dots. J Invest Dermatol 126: 1095–1101
164. Zhang LW, Yu WW, Colvin VL, Monteiro-Riviere NA (2008) Biological interactions of quantum dot nanoparticles in skin and in human epidermal keratinocytes. Toxicol Appl Pharmacol 228:200–211
165. Jeong SH, Kim JH, Yi SM, Lee JP, Kim JH, Sohn KH, Park KL, Kim M-K, Son SW (2010) Assessment of penetration of quantum dots through in vitro and in vivo human skin using the human skin equivalent model and the tape stripping method. Biochem Biophys Res Commun 394:612–615
166. Prow TW, Monteiro-Riviere NA, Inman AO, Grice JE, Chen X, Zhao X, Sanchez WH, Gierden A, Kendall MF, Zvyagin AV, Erdmann D, Riviere JE, Roberts MS (2012) Quantum dot penetration into viable human skin. Nanotoxicol 6:173–185
167. Labouta HI, Schneider M (2013) Interaction of inorganic nanoparticles with the skin barrier: current status and critical review. Nanomedicine 9:39–54

168. Ryman-Rasmussen JP, Riviere JE, Monteiro-Riviere NA (2006) Penetration of intact skin by quantum dots with diverse physicochemical properties. Toxicol Sci 91:159–165
169. Popovic Z, Liu W, Chauhan VP, Lee J, Wong C, Greytak AB, Insin N, Nocera DG, Fukumura D, Jain RK, Bawendi MG (2010) A nanoparticle size series for in vivo fluorescence imaging. Angew Chem Int Ed Engl 49:8649–8652
170. Cai W, Shin DW, Chen K, Gheysens O, Cao Q, Wang SX, Gambhir SS, Chen X (2006) Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects. Nano Lett 6:669–676
171. Bagalkot V, Zhang L, Levy-Nissenbaum E, Jon S, Kantoff PW, Langer R, Farokhzad OC (2007) Quantum dot-aptamer conjugates for synchronous cancer imaging, therapy, and sensing of drug delivery based on bi-fluorescence resonance energy transfer. Nano Lett 7:3065–3070
172. Wang CH, Hsu YS, Peng CA (2008) Quantum dots encapsulated with amphiphilic alginate as bioprobe for fast screening anti-dengue virus agents. Biosens Bioelectron 24:1018–1025
173. Fan HM, Olivo M, Shuter B, Yi JB, Bhuvaneswari R, Tan HR, Xing GC, Ng CT, Liu L, Lucky SS, Bay BH, Ding J (2010) Quantum dot capped magnetite nanorings as high performance nanoprobe for multiphoton fluorescence and magnetic resonance imaging. J Am Chem Soc 132:14803–14811
174. Nikitin MP, Zdobnova TA, Lukash SV, Stremovskiy OA, Deyev SM (2010) Protein-assisted self-assembly of multifunctional nanoparticles. Proc Natl Acad Sci U S A 107:5827–5832
175. Chen T, Zhao T, Wei D, Wei Y, Li Y, Zhang H (2013) Core-shell nanocarriers with zno quantum dots-conjugated au nanoparticle for tumor-targeted drug delivery. Carbohydr Polym 92:1124–1132
176. Dobson J (2008) Remote control of cellular behaviour with magnetic nanoparticles. Nat Nanotechnol 3:139–143
177. El Haj AJ, Glossop JR, Sura HS, Lees MR, Hu B, Wolbank S, Van Griensven M, Redl H, Dobson J (2012) An in vitro model of mesenchymal stem cell targeting using magnetic particle labelling. J Tissue Eng Regen Med. doi:10.1002/term.1636
178. Hughes S, Mcbain S, Dobson J, El Haj AJ (2008) Selective activation of mechanosensitive ion channels using magnetic particles. J Roy Soc Interface 5:855–863
179. Jain TK, Richey J, Strand M, Leslie-Pelecky DL, Flask CA, Labhasetwar V (2008) Magnetic nanoparticles with dual functional properties: drug delivery and magnetic resonance imaging. Biomaterials 29:4012–4021
180. Yu MK, Jeong YY, Park J, Park S, Kim JW, Min JJ, Kim K, Jon S (2008) Drug-loaded superparamagnetic iron oxide nanoparticles for combined cancer imaging and therapy in vivo. Angew Chem Int Ed Engl 47:5362–5365
181. Das M, Mishra D, Dhak P, Gupta S, Maiti TK, Basak A, Pramanik P (2009) Biofunctionalized, phosphonate-grafted, ultrasmall iron oxide nanoparticles for combined targeted cancer therapy and multimodal imaging. Small 5:2883–2893
182. Maeng JH, Lee DH, Jung KH, Bae YH, Park IS, Jeong S, Jeon YS, Shim CK, Kim W, Kim J, Lee J, Lee YM, Kim JH, Kim WH, Hong SS (2010) Multifunctional doxorubicin loaded superparamagnetic iron oxide nanoparticles for chemotherapy and magnetic resonance imaging in liver cancer. Biomaterials 31:4995–5006
183. Wang YX, Hussain SM, Krestin GP (2001) Superparamagnetic iron oxide contrast agents: physicochemical characteristics and applications in mr imaging. Eur Radiol 11:2319–2331
184. Jain TK, Morales MA, Sahoo SK, Leslie-Pelecky DL, Labhasetwar V (2005) Iron oxide nanoparticles for sustained delivery of anticancer agents. Mol Pharm 2:194–205
185. Shangary S, Qin D, Mceachern D, Liu M, Miller RS, Qiu S, Nikolovska-Coleska Z, Ding K, Wang G, Chen J, Bernard D, Zhang J, Lu Y, Gu Q, Shah RB, Pienta KJ, Ling X, Kang S, Guo M, Sun Y, Yang D, Wang S (2008) Temporal activation of p53 by a specific mdm2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proc Natl Acad Sci U S A 105:3933–3938
186. Zou P, Xu S, Povoski SP, Wang A, Johnson MA, Martin EW Jr, Subramaniam V, Xu R, Sun D (2009) Near-infrared fluorescence labeled anti-tag-72 monoclonal antibodies for tumor imaging in colorectal cancer xenograft mice. Mol Pharm 6:428–440

187. Jun YW, Huh YM, Choi JS, Lee JH, Song HT, Kim S, Yoon S, Kim KS, Shin JS, Suh JS, Cheon J (2005) Nanoscale size effect of magnetic nanocrystals and their utilization for cancer diagnosis via magnetic resonance imaging. J Am Chem Soc 127:5732–5733
188. Kas R, Sevinc E, Topal U, Acar HY (2010) A universal method for the preparation of magnetic and luminescent hybrid nanoparticles. J Phys Chem C 114:7758–7766
189. Zou P, Yu Y, Wang YA, Zhong Y, Welton A, Galban C, Wang S, Sun D (2010) Superparamagnetic iron oxide nanotheranostics for targeted cancer cell imaging and pH-dependent intracellular drug release. Mol Pharm 7:1974–1984
190. Kumar A, Jena PK, Behera S, Lockey RF, Mohapatra S (2010) Multifunctional magnetic nanoparticles for targeted delivery. Nanomedicine 6:64–69
191. Liong M, Lu J, Kovochich M, Xia T, Ruehm SG, Nel AE, Tamanoi F, Zink JI (2008) Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery. ACS Nano 2:889–896
192. Sudimack J, Lee RJ (2000) Targeted drug delivery via the folate receptor. Adv Drug Deliv Rev 41:147–162
193. Lee RJ, Low PS (1994) Delivery of liposomes into cultured kb cells via folate receptor-mediated endocytosis. J Biol Chem 269:3198–3204
194. Soppimath KS, Liu LH, Seow WY, Liu SQ, Powell R, Chan P, Yang YY (2007) Multifunctional core/shell nanoparticles self-assembled from pH-induced thermosensitive polymers for targeted intracellular anticancer drug delivery. Adv Funct Mater 17:355–362
195. Fan Z, Senapati D, Singh AK, Ray PC (2013) Theranostic magnetic core-plasmonic shell star shape nanoparticle for the isolation of targeted rare tumor cells from whole blood, fluorescence imaging, and photothermal destruction of cancer. Mol Pharm 10:857–866
196. Danhier F, Feron O, Préat V (2010) To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. J Control Release 148: 135–146
197. Tarn D, Ashley CE, Xue M, Carnes EC, Zink JI, Brinker CJ (2013) Mesoporous silica nanoparticle nanocarriers: biofunctionality and biocompatibility. Res. EPub ahead of print, Acc Chem
198. Kale A, Bao Y, Zhou Z, Prevelige PE, Gupta A (2013) Directed self-assembly of cds quantum dots on bacteriophage p22 coat protein templates. Nanotechnology 24:045603. doi:10.1088/0957-4484/24/4/045603
199. Hemmer E, Takeshita H, Yamano T, Fujiki T, Kohl Y, Low K, Venkatachalam N, Hyodo H, Kishimoto H, Soga K (2012) In vitro and in vivo investigations of upconversion and nir emitting gd(2)o(3):Er(3)(+), yb(3)(+) nanostructures for biomedical applications. J Mater Sci Mater Med 23:2399–2412
200. Marchuk K, Guo Y, Sun W, Vela J, Fang N (2012) High-precision tracking with non-blinking quantum dots resolves nanoscale vertical displacement. J Am Chem Soc 134:6108–6111
201. Riedinger A, Pernia Leal M, Deka SR, George C, Franchini IR, Falqui A, Cingolani R, Pellegrino T (2011) "Nanohybrids" based on pH-responsive hydrogels and inorganic nanoparticles for drug delivery and sensor applications. Nano Lett 11:3136–3141
202. Manabe N, Hoshino A, Yi-Qiang L, Goto T, Kato N, Yamamoto S (2006) Quantum dot as a drug tracer in vivo. IEEE Trans Nanobioscience 5:263–267
203. Xiao J, Wu M, Kai G, Wang F, Cao H, Yu X (2011) Zno-zns qds interfacial heterostructure for drug and food delivery application: enhancement of the binding affinities of flavonoid aglycones to bovine serum albumin. Nanomedicine 7:850–858
204. Ho YP, Chen HH, Leong KW, Wang TH (2006) Evaluating the intracellular stability and unpacking of DNA nanocomplexes by quantum dots-fret. J Control Release 116:83–89
205. Tan WB, Jiang S, Zhang Y (2007) Quantum-dot based nanoparticles for targeted silencing of her2/neu gene via rna interference. Biomaterials 28:1565–1571
206. Zaman MB, Baral TN, Jakubek ZJ, Zhang J, Wu X, Lai E, Whitfield D, Yu K (2011) Single-domain antibody bioconjugated near-IR quantum dots for targeted cellular imaging of pancreatic cancer. J Nanosci Nanotechnol 11:3757–3763

Chapter 19
Carbon-Based Nanomaterials for Targeted Drug Delivery and Imaging

Vivek S. Thakare, D'Arcy Prendergast, Giorgia Pastorin, and Sanyog Jain

19.1 Introduction

Despite numerous advances in the field of cancer biology and therapeutics, there remain considerable challenges in cancer therapy. The major hurdles are tissue selectivity of the treatment and target resistance to the drug action. The lack of selectivity for the tumor tissues results in debilitating side effects limiting patient compliance, whereas multidrug resistance (MDR) has been ascribed to the activity of P-glycoprotein (P-gp), which recognizes and effluxes the drug out of the cell [1]. Many efforts are being steered to selectively target the cancerous tissue with minimal damage to normal tissue; however, this is still a distant dream. The realization of such a system would require the identification of suitable biomarkers for neoplastic disease, the employment of these markers in screening and early detection and the development of a system for the biomarker-targeted delivery of multiple therapeutic agents, which will essentially bypass the normal tissue [2]. Several nanotechnology-based products have been approved for clinical use and many are in the clinical phases, namely, polymeric micro/nanoparticles, liposomes, and polymer–drug conjugates. New delivery systems have been continuously explored to improve the pharmacological performance of anticancer drugs and carbon nanotubes (CNTs) are no exception to this, as they have been envisaged as potential transporters for cancer therapy and diagnosis. CNTs are a unique class of

V.S. Thakare • S. Jain (✉)
Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Mohali, Punjab, India
e-mail: sanyogjain@niper.ac.in; sanyogjain@rediffmail.com

D. Prendergast • G. Pastorin
Department of Pharmacy, National University of Singapore, Singapore, Singapore

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_19

nanomaterials belonging to the fullerene family of carbon allotropes which are cylindrical in shape [3]. The unique physicochemical properties and easily manipulated surface of CNTs have led to diverse functionalization strategies, evident from the surge in the number of publications in this interesting arena. Compared to nanocarriers like liposomes/micelles (1960s) and nanoparticles/dendrimers (1980s) which emerged a few decades ago, CNTs are relatively new in biomedical research, and hence, we attempt to summarize the work done so far with CNTs in the field of cancer therapy and diagnosis [4].

19.2 Carbon Nanotubes: Attributes and Functionalization Strategies

Simply put, CNTs are hollow cylinders formed by rolling single or multiple layers of graphene sheets into seamless cylinders which consist of hexagonal arrangement of sp^2-hybridized carbon atoms (C-C distance of ~1.4 Å). These cylindrical structures exist in two forms: single-walled carbon nanotubes (SWCNTs) which are composed of a single cylindrical graphene layer capped at both ends in a hemispherical arrangement of carbon networks and multiwalled carbon nanotubes (MWCNTs) consisting of several concentric SWCNTs present within each other. The capping/closure of the cylinder in SWCNTs is effected by pentagonal and heptagonal C-C structures that provide hemispherical geometry during the growth process. Depending on the synthesis process, MWCNTs are comprised of several concentric cylinders of graphitic shells with an average interlayer separation of about 0.34 nm, each one forming a SWCNT resulting in a larger outer diameter (2.5–100 nm) than SWCNTs (0.6–2.4 nm). SWCNTs have a better defined diameter, whereas MWCNTs are more likely to have structural defects, resulting in a less stable nanostructure. However, MWCNTs continue to feature in many publications due to their ease of processing and the lack of conclusive advantages of SWCNTs relative to MWCNTs [5].

CNTs possess very high surface areas with SWCNTs having a typical surface area of ~1,300 m^2/g, whereas MWCNTs generally have surface areas of a few 100 m^2/g, based on theoretical assumptions. However, the available surface area is dependent on the length, diameter, and degree of bundling owing to which the surface area of most samples of SWCNT is dramatically lowered to ~300 m^2/g, or less, which is still a very high value [6, 7].

CNTs are available in a gamut of lengths and diameters depending on the synthetic procedure. Since, both SWCNTs and MWCNTs have a very strong tendency to cling together in ropes owing to attractive van der Waals forces analogous to the forces that bind sheets of graphite, they exist in bundles that contain many nanotubes and can be considerably longer and wider than the nanotubes from which they are formed. This bundling phenomenon could have important toxicological consequences in the context of biomedical applications [8, 9]. The solubilization of pristine CNTs in aqueous solvents presents a significant hurdle in realizing their potential as pharmaceutical excipients because of the hydrophobic character of the

graphene sidewalls, coupled with the strong p-p interactions between the individual tubes that result in bundling. As for any nanomaterial, the solubility/dispersibility of CNTs in aqueous solvent is a prerequisite for biocompatibility; hence CNT composites intended for therapeutic applications are expected to meet this basic requirement. To successfully disperse CNTs the dispersing medium should have the capability to wet the hydrophobic tube surfaces as well as be able to modify the tube surfaces to decrease tube aggregation. Moreover, it is essential that the CNT dispersions be uniform and stable to obtain accurate concentration data. Four basic approaches have been used to obtain dispersion: (a) surfactant-assisted dispersion, (b) solvent dispersion, (c) functionalization of CNT sidewalls, and (d) biomolecular dispersion [10]. In addition to decreasing cytotoxicity and improving biocompatibility, functionalization engenders an opportunity to append molecules for drug, protein or gene delivery [11]. The following sections briefly describe the widely used strategies for CNT functionalization.

One of the distinct features of CNTs is their capacity to form supramolecular complexes with polycyclic aromatic molecules through p–p stacking [1]. Several anticancer drugs as well as large polymeric anticancer molecules can be adsorbed on to the surface of pristine CNTs. The forces that govern such binding are the stacking interactions between the hydrophobic moiety of the adsorbed molecules and the hydrophobic surface of CNT, also represented by π–π stacking interactions. Many anticancer drugs being hydrophobic in nature such forces will facilitate the loading of such drugs onto/into the CNTs. Ionic interactions are also likely to be present enabling adsorption of the molecules in the instances where the nanotube surface has been chemically altered with charged functional groups [12, 13].

Oxidative treatment is often employed to remove the carbonaceous impurities as well as clearing the metal surface from CNTs. However, treatment with strong acids solutions generates cut at the surface of CNTs and gives rise to carboxylic acid groups at the defect point, predominantly on the open ends (Fig. 19.1). Moreover, excessive surface defects can alter the electronic properties and destruct longer CNT to shorter ones. The surface generated –COOH groups can further be treated with $SOCl_2$/oxalyl chloride or carbodiimide to give reactive intermediates that have greater propensity towards reacting with other compounds [15, 16].

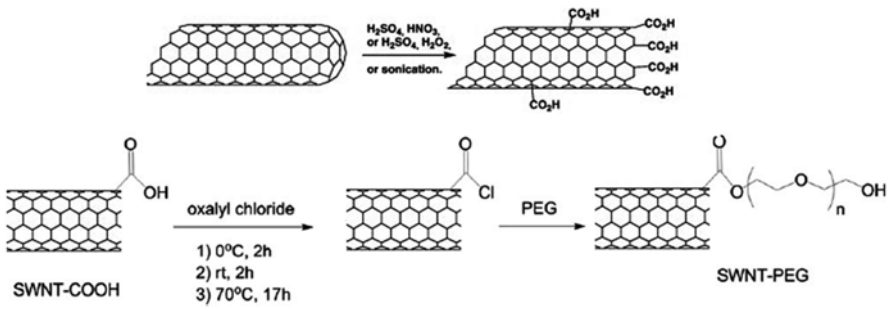

Fig. 19.1 Oxidation of pristine CNTs with different oxidizing agents to give –COOH functionalized CNTs and subsequent functionalization. Modified and reprinted after permission from ref. [14]

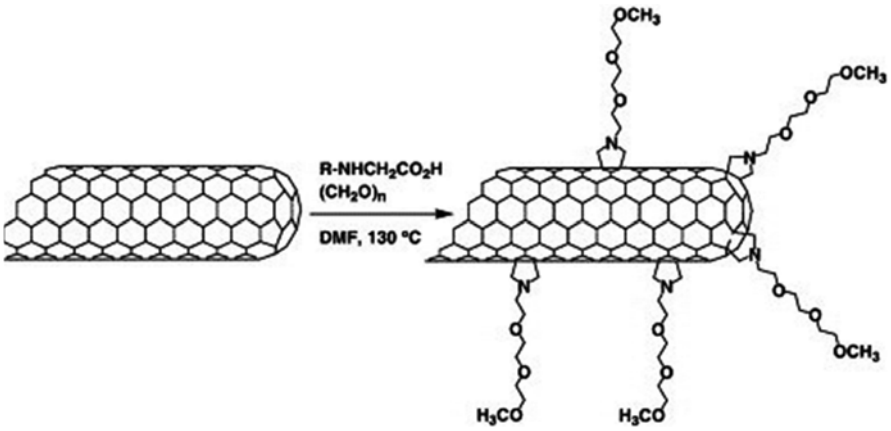

Fig. 19.2 Functionalization through 1,3-dipolar cycloaddition of azomethine ylides. Modified and reprinted after permission from ref. [17]

Azomethine ylides represent another avenue to alter the surface of CNTs which are produced by decarboxylation of immonium salts obtained from the condensation of α-amino acids with aldehydes or ketones. These compounds, when reacted with CNTs, can give CNTs fused with pyrrolidine rings with varied substituents depending on the structure of the α-amino acids and aldehydes employed. This approach has been used to functionalize CNTs with methotrexate [17]. The method is illustrated in the Fig. 19.2. Other methods of covalent functionalization have also been reported, but not yet used for anticancer drugs [15].

19.3 Filled Nanotubes for Cancer Therapeutics

CNTs can be considered to be nanobottles which can be filled with the therapeutic substances, to be emptied at the target site. By virtue of the interactions between CNTs and drug molecules, molecules can be loaded into/onto CNTs [18, 19]. Borowiak-Palen and coworkers have reported encapsulation of cisplatin, a small molecule into 1.3–1.6 nm diameter SWCNTs. Cisplatin is platinum (Pt)-based anticancer drug commonly used to treat various types of cancers, which acts through DNA cross-linking. However, it suffers from several undesirable side effects that limit its application. Encapsulation was carried out by dispersing SWCNTs and cisplatin in DMF through vigorous stirring for 48 h at 40 °C. Cisplatin can be also encapsulated inside SWCNTs (short and with open tips), which are treated with strong acid and annealed in a high vacuum environment [20]. The cisplatin incorporation into the tubes was confirmed with Raman and infrared spectroscopy and verified using high resolution transmission electron microscope. The cisplatin release was observed to occur for almost a week with maximum release being during 72 h encapsulation was 21 μg of drug per 100 μg of SWCNTs based on thermogravimetric analysis.

In vitro cytotoxicity studies using DU145 and PC3 cell lines revealed that the cell viability decreased with increase in the concentration of the CNT based nanovector whereas control, i.e., empty CNTs showed no effect on the cell viability. To understand the feasibility of interaction between nanotubes and drug molecules computational methods have been used [21]. Using elementary mechanics and applied mathematical modelling techniques, authors theorized that for cisplatin to be incorporated in CNTs, the CNTs must have a radius of at least 4.785 Å. Since, most of the nanotubes used have diameter greater than the above limit, it is apparent that cisplatin is likely to find its place inside nanotubes [22].

Recently, carbon nanotube–polymer composites have attracted much interest towards developing polymer-based nanocomposites which possess outstanding mechanical properties and multifunctional characteristics, for the range of applications (e.g., wind blades) [23]. Such an approach also renders multifunctionality to the biomedical application of CNTs. In an attempt to steer in this direction, hydrophilic polyacrylic acid (PAA)–MWCNT nanocomposites were prepared by appending polyacrylic acid (PAA) onto CNTs using radical polymerization, the ionic interactions between the surface –COOH groups and amino moieties of PAA were supposed to be involved in PAA coating on CNT and stabilization of nanocomposites from aggregation. Further, these composite was adsorbed with Fe_3O_4 magnetic nanoparticles (MN) by coprecipitation to render targeting under influence of magnetic field. Gemcitabine (GEM), an anticancer nucleoside with a low half-life was loaded into the above system with a loading efficiency of 62 %. Biodistribution studies revealed that after 3 h of subcutaneous injection of CNTs were absent in major organs like liver, kidney, heart, spleen and lungs, suggesting selective targeting of composites under the influence of a magnetic field. Authors also implicate the role of enhanced permeability and retention (EPR) effect, characteristic of the leaky vasculature in tumorous tissue [24].

Doxorubicin (DOX), a DNA intercalating anthracycline antibiotic, is a molecule of particular interest for drug delivery scientists, owing to its peculiar physicochemical and biological properties which renders it amenable to incorporation into nanoparticles. Doxil, DaunoXome, Myocet, and Caelyx represent the liposomal delivery systems for anthracyclines that have been approved for cancer therapy, however they suffer from superficial toxicity, “hand and foot syndrome” owing to prolonged blood circulation [25, 26]. The lower tissue selectivity and inefficient distribution of DOX can be overcome by using CNTs as drug carriers, due to their capability of binding to DOX on the surface or in their hollow space and ferrying it through cellular barriers. Liu et al. have created PEG-functionalized SWCNTs supramolecularly attached to DOX with a heavy drug loading of about 400 %, through exploiting the strong π–π and hydrophobic interactions between flat aromatic tetracyclic structure of DOX and aromatic surfaces of CNTs. These interactions were explained by monitoring the emission spectrum of doxorubicin by fluorescence spectrophotometry, in which fluorescence intensity of doxorubicin dramatically decreased owing to quenching as concentration of MWCNTs increased, (the concentration of PEG/Pluronic being constant at 1 % w/v) Cells treated with the equivalent demonstrated no difference compared to untreated cells with 100 % cell viability. The PEG functionalized doxorubicin–MWCNT complex demonstrated

enhanced cytotoxicity, relative to DOX alone, indicating that MWCNTs can enhance the delivery of doxorubicin and hence improve its cellular uptake. Moreover, pluronic coated MWCNTs and pluronic alone did not alter the viability of cells, establishing their inertness. However, in vivo studies are a critical validation of the system [27].

Several other research groups have developed doxorubicin loaded nanotubes with different features. Triply functionalized oxidized SWCNTs have been developed for delivery of doxorubicin, using a monoclonal antibody as a targeting moiety (linked via BSA as a linker), and a fluorescent marker as a imaging modality. The antibody-directed carrier recognizing carcinoembryonic antigen (CEA, which is a glycoprotein expressed only in cancer cells, especially adenocarcinoma such as colon cancers), was better internalized by CEA expressing WiDr colon cancer cells relative to untargeted CNT complex, as revealed by confocal microscopy. Subsequently, doxorubicin was released intracellularly, and translocated to the nucleus while the nanotubes remained in the cytoplasm [28]. On a similar note, Zhang et al. have fabricated doxorubicin loaded polysaccharide functionalized SWCNT, which were more biocompatible and dispersible than pristine CNTs. Surface wrapping of the carbon nanoparticles with naturally available polymers can be a potential approach towards enhancing biocompatibility of the inorganic particulates. The authors report to have effected this by sonication and stirring of chitosan–alginate solution with CNT which caused the polymer chain to wrap around the CNTs. These biopolymers were further tethered to folic acid (FA) through the amino group present on chitosan enabling targeting function. Chitosan being cationic and alginate anionic, they impart the coated SWCNTs with different surface electronic properties. Alginate-coated SWCNTs gave highest DOX loading, as the negative charges on alginate facilitated association with cationic DOX, converse to low level of DOX loading observed for CHI-coated CNTs due to mutual repulsion. The chitosan–alginate ratio can be optimized to exert the and better control over the release profiles of these CNT–polymer constructs. Moreover, because the hydrophilicity of DOX increases at acidic pH, causing it detach from nanotube surface, and since acidic pH is a hallmark of the tumor environment and intracellular lysosomes, a targeting effect was observed for these constructs. These carriers/complexes for doxorubicin could be more efficacious than the free drug and might result in reduced toxicity and side-effects with a probable decrease in the amount of the drug needed for therapy [29]. Although theses micro-fabricated constructs can serve as excellent theranostic tools, these complex systems need to be rigorously investigated to check the integrity of the SWCNT hybrids during their course through the biological milieu, which presents diverse degradative environments to externally administered objects. Supramolecular assemblies consisting of SWCNTs non-covalently functionalized by PL-PEG and doxorubicin (DOX) were prepared based on the π–π stacking interaction which provided for the attachment of DOX onto the CNT surface (50–60 % of doxorubicin), in which loading capacity was observed to be maximum at pH 9. SWCNTs without any DOX loading (PL-SWCNT) exhibited no toxic effect on cells whereas DOX-loaded SWCNTs (PL-SWCNT-DOX) induced significant U87 cancer cell death and cell apoptosis, indicative of the inert nature of carrier [30]. Several factors govern the adsorption and desorption of DOX from

CNTs like; loading and release pH, loading concentration, time of adsorption, dimensional attributes of CNTs, nature of functionalization on CNTs, temperature, and presence of competing substrates. DOX possesses an amine group in its structure and hence the physicochemical properties of DOX are highly sensitive to changes in environmental pH with unionized and hydrophobic form being the dominant species at neutral and basic pH (longer incubation time at basic pH should be avoided to prevent decomposition of DOX), whereas in acidic conditions, the amine on DOX can be protonated, increasing its hydrophilicity and solubility favoring desorption. This change in hydrophobicity is an important feature determining the loading and release of DOX from CNTs [31, 32]. The supramolecular approach in the sections above represents a novel, easily synthesized DOX carriers with extremely high drug-loading efficiency compared to those reported for conventional liposomes and dendrimer drug carriers.

Thus, the structural uniqueness of the doxorubicin makes such CNT-DOX based supramolecular complexes capable of self-targeting owing to the above mechanisms, making DOX the most widely explored drug molecule as far as the applications of CNTs in anticancer drug delivery is concerned. The above delivery strategies have been compounded through ligand based targeting like RGD peptide which exclusively targets integrin receptors (overexpressed on cancerous cells) (Fig. 19.3), providing multiple complementary targeting mechanisms for improved specificity [30].

Fig. 19.3 Schematic structure of doxorubicin loaded (based on stacking) SWCNTs functionalized with RGD at the termini of PEG. Reproduced from ref. [30]

It has been envisaged that antioxidants could play a seminal role in cancer therapy owing to their ability to combat oxidative stress. However, a majority of antioxidants have poor biopharmaceutical properties and suffer from poor solubility, making it difficult to administer them. As a result, several approaches have been adopted to enhance their delivery. Amino butylated hydroxy toluene (BHT) was ionically linked to PEGylated ultrashort SWCNTs, through simple stirring/incubation. The residual carboxylic acid groups on the oxidized CNT formed ionic complexes with the amine group of the BHT, a functional antioxidant. The complex was examined using ORAC (oxygen-radical absorbance capacity) assay, in which a fluorescent probe's loss of fluorescent intensity under the influence of oxygen radicals is measured. In the presence of oxygen-radical scavengers, the fluorescent intensity of probe remains unaffected until the radical scavenger is consumed. The radical scavenging ability of the CNT-BHT composite was found to be 1,240 times as that of Trolox, a vitamin E based radical scavenger used as control. The PEGylated SWCNT were found to be nontoxic on HRE and HepG2 liver cells when exposed for 24 h. However, when BHT was conjugated covalently to CNTs the antioxidant activity diminished, signifying that the conjugated systems should be selected carefully so as to preserve the active sites [12]. Table 19.1 summarizes various drug loaded CNT-based systems.

MWCNTs non-covalently functionalized with 1,2-distearoyl-phosphatidylethanolamine–methoxypolyethylene glycol-2000 conjugate (DSPE-mPEG 2000) were used to entrap methotrexate (MTX) [33]. MWCNT–mPEG–MTX, were characterized for particle size, loading efficiency, morphology, and in vitro release. The f-MWCNT was found to release MTX faster in acidic medium of pH 5.8 relative neutral pH 7.4, signifying role in cancer tissue targeting, which needs to be expanded upon with in vivo studies. On the similar lines, Das et al. physically loaded different chemotherapeutic agents, including MTX, on an array of differently functionalized MWCNTs viz. with PEG, HA, FA and ES [34]. Irrespective of the nature of functionalization the loading of MTX was around 30 % for all samples.

19.4 CNT Based Conjugated Systems for Cancer Targeting

Polymer–drug conjugates (PDC) are emerging drug delivery tools, with several polymeric conjugates advancing to the clinical trial stage. Polymer–drug conjugates afford distinct advantages over the corresponding parent drugs like fewer side effects, improved therapeutic efficacy, drug administration ease, and better patient compliance. Primarily, the polymeric conjugates exert their effect through enhanced permeability and retention, owing to their stealth macromolecular structure, which enables them to selectively accumulate in tumor tissue. Along with this, pathophysiologic factors at the tumor site, namely, enhanced permeability, poor venous drainage, acidic pH, and relatively high temperature enhance the pharmacological profile of PDC [35]. Conjugating CNTs with other moieties is a significant challenge owing to lack of homogeneity of dispersions and subsequent characterization of conjugates, but nevertheless are being explored for improvement in the cancer therapy.

Table 19.1 Summary of representative examples of CNT based cargos using simple drug loading (adapted and reprinted after permission from ref. [3])

Type of functionalization/ carrier system	Drug loaded/nature of loading	Physicochemical characterization	In vitro/in vivo system	Inference of study	Ref
Oxidized SWCNT	Cisplatin, Endohedral filling	Raman Spectroscopy, FTIR, HR-TEM, ICP, TGA	DU145 and PC3 cell lines	Dose dependent cell viability of drug loaded CNTs contrary to empty CNTs which did not kill cells.	[20]
Polyacrylic acid functionalized Fe_2O_3 based magnetic MWCNT	Gemcitabine, Endohedral filling along with entrapment in the polymer and metallic matrices	TEM and magnetometry.	SD Rats	Preferential accumulation and targeting in the lymphatic vessels under influence of magnetic field.	[24]
Pluronic F127 solubilized MWCNT	Doxorubicin, stacking interactions	TEM and fluorimetry	MCF-7 human breast cancer cells.	Enhanced cytotoxicity of doxorubicin–MWCNT complex over doxorubicin alone.	[27]
Folate targeted chitosan/alginate coated SWCNTs	Doxorubicin, stacking and entrapment in polymer matrices	TEM and UV–visible spectroscopy	HeLa cells	The system was found to be more selective and effective than the free drug due to targeting based on FA and release of DOX at acidic pH	[29]
mAB-BSA adsorbed SWCNTs	Doxorubicin, stacking	SEM, TEM, AFM, Raman spectroscopy, and TGA	WiDr colon cancer cells	Uptake study revealed that delivery efficiency was 100 %—all cells could take up the SWCNT complexes.	[28]
PL-PEG-RGD functionalized SWCNT	Doxorubicin, stacking	AFM, UV–Visible and fluorescence spectroscopy	MCF-7 breast cancer cells and U87MG human glioblastoma cancer cells	Enhanced uptake of Dox in case of integrin positive U87MG using RGD based targeting relative to integrin negative MCF-7 cells	[30]

Methotrexate (MTX) is a drug widely used against cancer; however, it suffers from low cellular uptake. Tethering of MTX to CNT is a promising approach to circumvent its limited cellular uptake by enhancing its internalization via the f-CNTs [36].

Pastorin et al. introduced two orthogonally protected amino groups on the sidewalls of CNTs and subsequently conjugated it with fluorescein isothiocyanate (FITC) and MTX using the 1, 3-dipolar cycloaddition of azomethine ylides. Epifluorescence and confocal microscopy studies revealed rapid internalization of the construct in Human Jurkat T lymphocytes in a dose and time dependent manner [36]. Aiming at tissue selective enzymatic cleavage, Samori and coworkers have covalently linked MTX to MWCNTs with two different linkers; tetrapeptide Gly-Leu-Phe-Gly (which can be selectively cleaved by proteases overexpressed in tumor cells), and the 6-hydroxyhexanoic ester (which is an esterase-sensitive hydrophobic spacer commonly used in other prodrug conjugate synthesis). It was observed that the cytotoxic activity of the conjugates in MCF-7 cells was influenced by the presence and the types of linker [37].

Paclitaxel (PTX), extracted from the Pacific Yew tree (*Taxus brevifolia*) belongs to a class of diterpene alkaloids called taxanes synthesized by plants of the Taxus genus. It is used for the treatment of ovarian cancer and is marketed under the trade name Taxol®. Docetaxel (DTX) is a semisynthetic analogue derived from a precursor found in the European Yew tree (*Taxus baccata*). These molecules promote microtubular aggregation and interfere with essential cellular functions like mitosis, cell transport, and cell motility leading to cell death. Taxanes have poor solubility in water compared to other anticancer drugs and hence excipients are necessary to increase its solubility, improve the bioavailability and limit their side effects. Current formulation (Taxol) encompasses cremophor as a solubilizer which has been shown to exhibit allergenic activity, necessitating the search for an alternative delivery system [38]. Unlike DOX, PTX does not possess aromatic structure and hence is not amenable to stacking interactions with CNTs. Nonetheless, PTX has been reported to be loaded on carbon nanotubes, potentially through hydrophobic interactions along with conjugation.

Liu and coworkers have conjugated PTX to branched polyethylene glycol phospholipid chain adsorbed on SWCNTs via a cleavable ester bond to obtain a water-soluble SWCNT-PTX conjugate, with a loading capacity of 150 PTX molecules per 100 nm length of nanotube. The SWCNT-PLPEG-PTX conjugate yielded higher efficacy in inhibiting tumor growth relative to Taxol alone in a murine 4T1 breast cancer model, indicating that active PTX was able to be released subsequent to ester cleavage in cells without any loss of pharmacological activity. Presence of PEG contributed to stealth effect causing prolonged blood circulation almost six-fold higher than Taxol along with tenfold higher tumor PTX uptake by SWCNT delivery, also likely mediated through enhanced permeability and retention (EPR effect) [39].

Analogous to the above studies, hyperbranched polycitric acid (PCA) was used to functionalize MWCNTs in order to increase their solubility, after which PTX was appended by esterification to the carboxyl groups of PCA. In vitro drug release study revealed faster release of PTX (independent of esterases) from the conjugates

in acidic pH, making it amicable to tumor tissue targeting. In vitro cytotoxicity tests concluded improved potency of PTX compared to carrier-free PTX in A549 lung and SKOV3 ovary cancer cell lines [40]. Yang et al. have hypothesized that nanotubes can be conferred magnetic properties (MNTs) through impregnation with magnetite (Fe_3O_4) nanoparticles on the inner surface of the nanotubes. Further, these MNT can be functionalized with phospholipid-polyethyleneglycol-folic acid (PL-PEG-FA) and loaded with chemotherapeutic agents, such as 5-fluorouracil (5-FU) and cisplatin through nanoprecipitation. Although this paper is an ideation, such a system can be steered by an externally placed magnet to target regional lymphatic nodes and hence is worthy of mention [41].

The platinum complexes used widely for cancer therapy are uncharged, *cis-configured*, square planar complexes with platinum in its +II oxidation state (Pt(II)). Structurally, they can be depicted as cis-[PtA2X2], A2—two monodentate or one bidentate ligand(s) with nitrogen donor atoms and X2—two monodentate or one bidentate anionic ligand(s). These compounds are DNA chelators and form intrastrand adducts that affect vital cellular processes like transcription and replication, and ultimately lead to apoptosis [42].

However, platinum-based anticancer drugs suffer from several drawbacks like poor blood circulation times, severe dose-limiting nephrotoxicity, neurotoxicity, and myelosuppression, arising from nonspecific target interactions. Because of unusually low size and intracellular DNA binding Platinum-based drugs are attractive candidates for prodrug strategy. A targeted platinum(IV) complex $[Pt(NH_3)_2Cl_2(O_2CCH_2CH_2CO_2H)(O_2CCH_2CH_2CONH\text{-}PEG\text{-}FA)]$ was conjugated to the surface of an amine-functionalized single-walled carbon nanotube (SWCNT-PL-PEG-NH_2) through several amide linkages to ferry it to the tumor cell where cisplatin is released upon intracellular reduction of Pt(IV) to Pt(II) after endocytosis. The SWCNT–cisplatin conjugate was found to be 8.6 times more active than cisplatin-based on the cell internalization studies owing to specific binding of conjugate to the folate receptors [43]. In the absence of the target ligand, SWCNT-conjugate as depicted in Fig. 19.5 was 2.5 times more toxic on NTera-2 cells relative to cisplatin [44].

Wei Wu et al. have synthesized a water-soluble prodrug of MWCNT-HCPT (hydroxy camptothecin) by covalently attaching HCPT to carboxy-MWCNTs with a linker diaminotriethylene glycol as the spacer between the two moieties following the derivation of HCPT with succinic anhydride at the 10-hydroxy position, with a drug loading of 16 %. The CNT construct was labelled with radioactive nuclide technetium-99 m and injected into the tail vein of mice to see the biodistribution. Single photon emission computed tomography imaging revealed that the complex was rapidly and strongly distributed to liver, spleen, and lung and weakly distributed into intestine, skin, and muscle. This biodistribution pattern was echoed in healthy non-tumor bearing mice. Moreover, radioactive intensity did not change significantly over time indicative of slow excretion and prolonged circulation time with half-life of around 3.6 h for CNT-HCPT complex, which was significantly longer than that reported for HCPT of only 30 min. Although the CNT based hybrid was not specific to tumors due to the absence of targeting moieties, it was found to be

enriched in tumors over the experimental duration of 22 h. Longer blood circulation and high tumor accumulation was observed in vivo with superior antitumor effect of the conjugates, almost twice in comparison to clinical HCPT injection and low toxicity to the living mice [45]. Polymer wrapping based solubilization through polymers like Phospholipid–PEG, chitosan, alginate, etc. and then functionalizing/conjugating them with drugs/ligands are not uncommon. However, Murakami et al. have exemplified a novel approach of dispersing carbon nanohorns (CNH) using DOX-PEG conjugate. The π–π stacking interactions between the nanohorn surface and DOX, indirectly provided for the attachment of PEG to CNH enhancing their dispersibility. Such an approach would also possibly be applicable for carbon nanotubes [46].

Dendrimers are highly branched, multiple-shaped synthetic macromolecules having diameter in the range of nanometers. They have distinct architecture and flexibility which include (a) a multivalent surface (nanoscaffolding), (b) interior shells, and (c) a core to which the dendrons are attached. These features along with vast exposed surface area enables good control of their dimension as well as conjugating different molecules such as therapeutic agents, targeting moieties, and fluorescent/imaging dyes. Although dendrimers are promising delivery tools, some dendrimeric systems have shown significant cytotoxicity and also drug very slowly, which might be overcome with the pH responsive polymers with high sensitivity and reduced toxicity. However, other than utility as delivery carriers, dendrimers have been explored as bioactive agents by Starpharma, which has led to a dendrimer-based microbicide (VivaGel) in the prevention of HIV and sexually transmitted infections (STI) [47, 48].

In an interesting study to integrate properties of CNT with dendrimers, Shi et al. used carboxyl MWCNTs that were covalently tethered to amine-terminated generation 5 poly(amidoamine) (PAMAM) dendrimers using EDC chemistry, which in turn were tagged with FITC and FA, and subsequently supramolecularly complexed with DOX. The dendrimer–CNT constructs was characterized using a gamut of techniques, namely, UV–Vis spectrometry, ^{1}H NMR, transmission electron microscopy (TEM), zeta potential measurements, and thermogravimetric analysis (TGA) and was found to be stable and biocompatible. The DOX loaded dendrimer–CNT constructs were selectively internalized by human epithelial carcinoma KB cells with overexpressed FRs resulting in more cytotoxicity than in KB cells with low levels of FRs [32]. The above study reveals that the attachment of dendrimers amplifies the number of the functional groups present on the nanotubes, which not only help conjugate large amounts of drug but also load drugs noncovalently in the cavities/core of the dendrimers [49]. A summary of different CNT based conjugated systems has been presented in Table 19.2.

Table 19.2 Representative CNT based conjugated systems (adapted and reprinted after permission from ref. [3])

Type of CNTs	Nature of functionalization	Physicochemical characterization	In vitro/in vivo system	Reference
SWCNTs	Paclitaxel conjugated to DSPE-PEG5000 (type of phospholipid)	UV–Visible Spectroscopy, Radiological studies, and DLS	4T1 tumor (breast cancer cell) induced mice	[39]
MWCNTs	Methotrexate conjugation through azomethine ylide chemistry (1,3 cycloaddition)	ATR-FT-IR, ^{1}H and ^{13}C NMR, TEM	Human Jurkat T lymphocytes	[37]
MWCNTs	Conjugation to PAMAM (G5) dendrimers	UV–Visible Spectroscopy, TEM, DLS, TGA and NMR	KB cells human epithelial carcinoma cell line	[49]
SWCNTs	Cisplatin conjugated PL-PEG	Cyclic voltammetry, AAS	NTera-2	[44]
SWCNTs	Cisplatin and folate conjugated PL-PEG	^{1}H NMR, ^{195}Pt NMR, LC-MS, HRMS, Cyclic voltammetry	KB and NTera-2 Cells	[43]
MWCNTs	10-hydroxycamptothecin (HCPT) covalently conjugated to CNTs using hydrophilic diaminotriethylene glycol as a linker	UV–Vis spectroscopy, ^{1}H, ^{13}C NMR, ESI-MS and XPS	H22 tumor induced mice	[45]
SWCNTs	BHT (butylated hydroxyl toluene) conjugated to PEG conjugated CNTs	IR, ^{1}H and ^{13}C NMR, and HRMS	ORAC assay	[12]
Carbon Nanohorns (CNHs)	Solubilization using Doxorubicin–PEG conjugate based on stacking interactins	GPC, TEM, DLS, and UV–Visible Spectroscopy	NCI-H460 human non-small-cell lung cancer cells	[46]

19.5 CNTs in Protein Based Targeting and Protein Delivery

The transporting capabilities of carbon nanotubes along with functional proteins have opened exciting avenues for drug delivery and cancer therapy. Tumor cell vaccines (TCV) are inactivated cancer cells or dendritic cells entailing tumor antigens which when administered incite immune response against the tumor itself [50]. In an attempt to explore CNT-based antitumor immunotherapy, tumor lysate proteins were covalently attached to oxidized MWCNTs via an amide bond. The study reveals how CNTs can be used effectively for augmenting the immune responses to cancerous cells.

Weng et al. have successfully conjugated recombined ricin A chain protein toxin (RTA) to MWCNTs to target tumor cells via CNTs based molecular transporters.

The RTA–MWCNT conjugate traversed the cell membrane across various cell lines, namely, L-929, HL7702, MCF-7, HeLa, and COS-7, causing cell death at the rate three times higher than that induced by RTA alone. Coupling this complex, to HER2 (antibody) further enhanced the tissue recognition through HER2/neu receptor causing targeted destruction of breast cancer cells [47]. In a similar context, Xiao et al. have developed a system that integrates properties of anti-HER2 IgY antibody with the unique properties of SWCNTs. As proteins are highly unstable in harsh environments, the conjugation approach utilized should be soft. One such direction is aqueous-based EDC chemistry, which the group has adopted to synthesize covalent HER2 IgY-SWCNT conjugates. Cell internalization studies were done using Raman spectroscopy, which exhibited Raman signals from the SK-BR-3 cells treated with the complex at the single cell level in comparison to control cells. Furthermore, thermal ablation could be selectively induced on HER2-expressing SK-BR-3 cells without harming HER2-free MCF-7 cells, through NIR (near infrared) irradiation, potentially due to a SWCNT-mediated steep increase in cellular temperatures [51].

SWCNTs have been demonstrated to act as antigen-presenting carriers, for the weakly immunogenic peptide: Wilm's tumor protein (WT1), which has been implicated as a vaccine candidate for human leukemias and is currently in human clinical trials. The covalent SWCNT–WT1 was rapidly taken up by antigen presenting cells, in a dose-dependent manner in vitro which was upheld by in vivo immunization of BALB/c mice. When administered in mice, SWCNT–WT1 and an adjuvant triggered a specific humoral immune response which was not evident in treatment with the peptide alone or with the peptide mixed with the adjuvant. Thus, SWCNTs can be used to improve the vaccine therapy through its carrier/adjuvant effect for weakly immunogenic proteins [52].

Anticancer agent cisplatin and epidermal growth factor (EGF) were conjugated to SWCNTs to specifically target squamous cancer as shown in the Fig. 19.4. SWNT-cisplatin without EGF served as a non-targeted control. Qdot (linked covalently) luminescence and confocal microscopy was used for in vitro imaging studies using head and neck squamous carcinoma cells (HNSCC) overexpressing EGF receptors(EGFR). The study revealed that SWNT-Qdot-EGF bioconjugates were internalized rapidly into the cancer cells. However, control cells demonstrated limited uptake as the uptake was blocked by siRNA knockdown of EGFR in cancer cells, revealing the importance of EGF binding. Three-color, two-photon intravital video that was used for imaging in vivo showed that when live mice was injected with SWNT-Qdot-EGF, SWNT-Qdot-EGF was selectively taken up by HNSCC tumors causing its rapid regression, but SWNT-Qdot controls with no EGF were cleared from the tumor region in <20 min. SWNT_cisplatin_EGF treated HNSCC cells were also killed selectively, while control systems that did not feature EGF-EGFR binding led to no control on cell proliferation [53].

In a study by McDevitt et al., side wall-functionalized, water-soluble CNTs were used to synthesize tumor-targeting CNT constructs by covalently attaching multiple copies of tumor-specific monoclonal antibodies, radiometal-ion chelates, and fluorescent probes. These nanoconstructs were found to be specifically reactive with the human cancer cells. They were designed to target disseminated human lymphoma, which was compounded by and in vitro studies using flow cytometry and cell-based

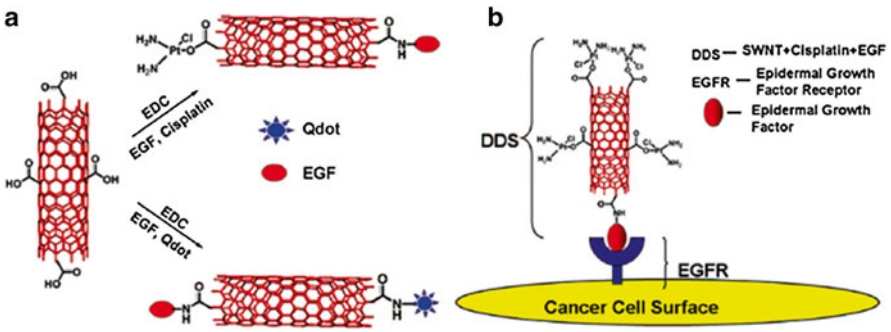

Fig. 19.4 Nanotube-based delivery system. (**a**) Illustration of EDC based coupling reactions used to attach EGF, cisplatin, and Qdots onto carboxylated SWCNTs (in *red*) (**b**) Schematic showing EGF and cisplatin conjugated SWCNTs targeting the cell surface EGFR on a single HNSCC cell. [EDC—1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, EGF—Epidermal Growth factor, Qdot—Quantum Dots]. Reprinted after Permission from ref. [53]

immunoreactivity assays [54]. In a pioneering study, Chakravarty et al. used Biotinylated polar lipids [1,2-distearoyl sn-glycero-3-phosphoethanolamine-*N*-{bio tinyl(polyethyleneglycol) 2000} {DSPE-PEG(2000)-biotin}], to prepare stable, biocompatible, noncytotoxic CNT dispersions that were then attached neutralite avidin-derivatized mAbs directed against either human CD22 or CD25. Thus, $CD22^{+}CD25^{-}$Daudi cells and $CD22^{-}CD25^{+}$ activated peripheral blood mononuclear cells were bound only to the anti-CD22 mAb and anti-CD25 mAb respectively which were coupled to CNTs. Further, exposure to NIR light generated heat caused photothermal ablation of targeted cells only [55]. The concept of photothermal ablation has been discussed later. Similar approach was reported by Kam et al., wherein SWNTs functionalized with PL-PEG-FA(Phospholipid–Polyethylene glycol–Folic acid) were used to achieve tumor cell targeting [56].

Tumor lysate protein (TLP) is a mixture of various tumor proteins that can be implicated in large number of tumors and patients, irrespective of the genetic makeup of the tumors. However, the lack of defined tumor markers, such as target antigens, led to difficulty in assessing the antibody responses. Further, since the nature of the immunogens was not known, tumor rejection, tumor growth retardation, or prolonged survival of the immunized mice were used to study the efficacy of therapy involving the tumor lysate proteins rather than by antibody production. CNTs have thus been used to improve the anticancer immune response of TLP against multiple tumors [50].

Integrin $\alpha_v\beta_3$ receptors/proteins are widely expressed on the cell surface of cancer cells and play critical role in tumor angiogenesis and metastasis [28], making RGD/arginine–glycine–aspartic acid peptide, a potent integrin $\alpha_v\beta_3$ antagonist, a ligand of choice for efficient tumor targeting of nanocarriers. RGD was conjugated through amino group on PL–PEG-functionalized SWNTs with diameter and length, 1–5 nm and 100–300 nm respectively. Furthermore ^{64}Cu using DOTA was used as chelating agent to radiolabel the system A high tumor accumulation (13 % ID/g over long periods >24 h), due to enhanced tumour binding affinity, was attributed to

polyvalency effect owing to unique one-dimensional shape and flexible structure of SWNTs. The Raman signals of SWCNTs could directly probe the presence of nanotubes in mice tissues coupled with the radiolabel-based results. No obvious toxicity, weight loss, or fatigue was observed for several months, with many mice injected with SWNT–PEG at dosages up to 2 mg/kg over monitoring periods [28]. Retention of radioactivity in mice explicates slow excretion of SWNTs, hence the long-term fate of SWNT–PEG injected into mice requires further investigation [57]. As a targeting ligand $\alpha_v\beta_3$ mAb is advantageous in terms of high specificity towards antigen $\alpha_v\beta_3$ and greater in vivo stability relative to RGD peptide. Thus, based on similar lines as discussed above, $\alpha_v\beta_3$ monoclonal antibody was also used to target the SWCNT using PL-PEG. However, in case of $\alpha_v\beta_3$ mAb, SWCNT-PL-PEG was first linked to protein A, to which the antibody was attached. The above conjugate was also tagged with fluorescent moiety generating a macromolecular structure. Although cell culture based studies in U87MG (human glioblastoma cancer cells) and MCF-7 (human breast cancer cells) reveal good targeting efficiency, the behaviour of such a macromolecular structure inside the complex biological system (in vivo) needs to be traced to confirm its value in cancer therapy [58].

19.6 Gene Delivery to Cancer Cells Using CNTs

Gene therapy has emerged as a most promising method in cancer treatment, which involves the treatment of both genetic and infectious diseases by introducing genetic materials which have therapeutic effects. This method is expected to be an alternative method to traditional chemotherapy. Traditionally used viral vector-based delivery achieves a high level of gene expression, but is limited by its immunogenicity, oncogenic potential, and inflammation and packaging capacity. Hence, multiple nonviral delivery systems have been introduced to efficiently deliver siRNA, including chemical modification of siRNA, cationic polymers, liposomes, cationic lipids, dendrimers, cell-penetrating peptides, and so on. However, these tools suffer from inefficient gene expression relative to viral vectors, due to their poor capability in reaching and crossing the nuclear membrane [59, 60]. Many years of research have led to the emergence of many viral and nonviral vectors, and carbon nanotubes have recently been recognized as nucleic acid carriers owing to their unusual properties [61].

The manner in which the CNTs interact with nucleic acids has been extensively studied: single-stranded DNA and oxidized CNTs interact in solution, under sonication, to form a charged hybrid complex, DNA/SWCNT. In conjunction to the ionic interactions, nucleic acids bind to nanotubes through π–π stacking interactions between the aromatic nucleobases and the nanotubes' graphene side walls. By acting as a supportive skeleton, nanotubes spontaneously orient DNA molecules around them, so as to wrap DNA around its cylindrical axis, which has been confirmed through AFM and spectroscopic studies. The sugar and phosphate groups remaining at the periphery relative to the bases help enhance the dispersibility of CNTs. These systems have been explored for diagnostic and gene delivery applications [62].

In an attempt to combine the properties of two cationic polymers, Hexamethylenediamine (HMDA) was covalently linked to the oxidized CNTs, whereas poly (diallyldimethyl-ammonium) chloride (PDDA) was bound by non-covalent interactions. Extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) genes were loaded onto HMDA-PDDA-SWCNT and its effect was studied on isolated rat heart cells, which exhibited better transfection efficiency and suppression of the expression levels of both ERK1 and ERK2 by nearly 80 %. These complexes were also found to be biocompatible at concentrations of 10 mg/L as it did not induce any significant cytotoxicity [63]. Likewise, in a study led by Pantarotto et al., positive charge-bearing ammonium f-CNTs were used to transfect plasmids (β-galactosidase encoded gene, β-gal) into HeLa and CHO cell lines and electrostatic forces were involved in the interaction between cationic CNTs and anionic DNA. Transfection efficacy was found to be enhanced ten times more in the case of the CNT-DNA complex relative to DNA alone. The transfection efficiency was observed to be proportional to CNT/DNA charge ratio with the maximum efficiency being observed at 6:1[64].

Yang et al., have synthesized cationic SWCNTs using 1,6-diaminohexane to present interfering RNA (siRNA) into the antigen-presenting dendritic cells in vivo. It was observed that siRNA:SWCNT complexes were preferentially taken up by splenic CD11c+ DCs, CD11b+ cells, and Gr-1+CD11b+ cells comprising DCs, macrophages, and other myeloid cells. The CNT-based gene complex could silence suppressor of cytokine signaling 1 (*SOCS1*) expression and thereby retard B16 tumor growth in mice [68]. Both antisense oligonucleotides and small interfering RNA (siRNA) are very promising therapeutic tools based on the fact that they inhibit protein expression, potentially blocking many cellular pathways and hence cancer therapy is one of the potential applications, when targeting oncogenes or genes involved in angiogenesis/chemotherapy resistance. Likewise, telomerase reverse transcriptase (TERT) plays a crucial role in tumor growth and development through the maintenance of telomere structure. siRNA delivered via SWCNT-CONH-$(CH_2)_6$-$NH_3^+Cl^-$ complexes was found to suppress the expression of TERT and prevent growth and proliferation of tumor cells both in vitro and in mouse models. At the end of 48 h, the cells treated with TERT siRNA:SWCNT almost exhibited complete inhibition of proliferation in murine tumour models [65].

Carbon nanotubes are amenable to surface modifications using charge-based layer-by-layer assembling. Carboxy or oxidized nanotubes which are negatively charged can be coated with widely reported cationic polymers (polyelectrolytes) like polyethylenimine (PEI), poly(diallyldimethylammonium) chloride (PDDA), poly(amidoamine) (PAMAM) dendrimers, and chitosan to impart cationic surface to CNTs. Such constructs were linked to suppression of telomerase expression using ASODN (antisense oligodeoxynucleotides) and represent a novel approach in cancer therapy as activity of telomerase is unusually high in 90 % of cancer cells compared to normal cells. Mercaptoacetic acid-capped CdTe quantum dots as fluorescent probes were linked to ASODN, which was in turn complexed with PEI-functionalized MWCNTs based on ionic interactions. The ASODN-PEI (polyethyleneimine)-MWCNTs ionic complex exhibited efficient intracellular uptake, strong nuclear

localization of ASODNs relative to ASODN in HeLa cells based on confocal and flow-cytometric studies. The above direction was corroborated through overexpression of enhanced green fluorescence protein (EGFP) upon the transfection of EGFP gene using PEI-MWCNT. The authors hypothesize that these vehicles protect nucleic acids from enzyme degradation and also exhibit enhanced proton-sponge effects due to the PEI coating on the surface of MWCNTs [13].

19.7 Light-Induced Thermal Ablation of Cancer Cells Using CNTs

The selective thermal ablation of malignant tissue is of prime importance in cancer research and tenders a viable alternative treatment option when surgical resection is not possible. As a result, photothermal therapies for cancer have been widely explored as a minimally invasive treatment option relative to other therapies [66]. At temperatures above 40 °C cell death occurs due to irreversible protein denaturation or membrane damage [67]. Carbon nanotubes possess outstanding attributes for the development of state-of-the-art photothermal agents, the foremost being their ability to generate heat under NIR radiation (700–1,100 nm), which has recently attracted much interest. Compared to other wavelengths of light, the transmission of NIR through the body is poorly attenuated by biological systems and as a result, body tissue does not absorb NIR radiation, a property which can be exploited in stimuli-sensitive targeted cancer therapy [68, 69]. Due to the arrangement of the carbon atoms in a confined nanometer-sized volume and shape (high aspect ratio—length/diameter), the absorption of electromagnetic energy is enhanced thereby generating heat [70]. Electronic transitions from the first or second van Hove singularities results in strong optical absorbance demonstrated by the nanotubes which impart them the intrinsic property of photothermal and photoacoustic effects. The van Hove-like singularity in the density of states (DOS) moves towards the top of the valence band, thereby enhancing the effective DOS near the Fermi energy and augmenting the electron–phonon interaction, so as to increase the temperature of the nanotubes [71, 72].

Subsequent to exposure to NIR, CNTs enter an excited state and release vibrational energy that is transformed into heat, which is then transferred to the surrounding matrix, causing cellular death, which is more pronounced in mammalian cancer cells as they are more sensitive to heat and apoptosis than other cells [73]. Although there is one group which has reported hyperthermia induced through radiofrequency radiation using CNTs, it is less widely explored and is less efficient than NIR excitation. RF ablation (RFA) is currently used in clinical practice to treat some malignant tumors but suffers from several drawbacks: (a) the insertion of needle electrodes directly into the tumor(s), (b) incomplete tumor destruction, and (c) thermal necrosis in normal tissues surrounding the needle electrode and associated complications. RF energy fields have better tissue penetration which can be augmented by use of nanotubes for noninvasive treatment of malignant tumors at any site in the body since it converts RF energy into heat locally at the site of malignant cells [74]. Gannon et al. have studied photothermal effects of SWCNTs functionalized with

polyphenylene ethynylene using a 13.56-MHz RF field. The targeted carrier was able to produce a noninvasive, selective, and concentration-dependent thermal destruction in vitro of human cancer cells which had internalized SWCNTs. Above results were corroborated by an intratumoral injection of SWCNTs into hepatic VX2 tumors followed by immediate RF field treatment which was well tolerated by rabbits. Post 48 h, all SWCNT-treated tumors exhibited complete tissue necrosis, whereas the control group, viz., tumors that were treated with RF without SWCNTs and those injected with SWCNTs but were not treated with RF, remained completely viable, indicative of the lower toxicity potential of f-SWCNTs [74].

The effect of the length of nitrogen doped, multiwalled carbon nanotubes (CNx-MWCNT) on the kidney cancer cells under influence of NIR radiation for photoablative destruction was studied by Carroll et al. The cytotoxicity at low radiation doses was found to be proportional to the length of the nanotubes, with effective NIR coupling witnessed at nanotube lengths exceeding half the wavelength of the incident radiation, which supports classical antenna theory. The presence of dopants often leads to structural defects across the length of nanotubes due to C=C bond breakages, which in turn enhances its heating efficiency. Destruction of the tumor cells occurs effectively when suspended nanotubes lie within their heating radial distance from the cells (R_{ZONK}—"zone of nanotube killing"). MWCNTs are believed to be more advantageous over SWCNTs due to (a) Absorption of electromagnetic energy increased threefold, (b) activation by broader band widths of electromagnetic radiation, (c) ability to kill cells present within R_{ZONK}, and (d) enhanced chemical and thermal stability [75]. Burke et al., injected Pluronic F127 (1 %) solubilized suspension of MWCNTs intratumorally in mice to demonstrate that MWCNTs alone have the potential to significantly ablate the cancerous tissue. A 100 μg dose of MWCNTs was successful in complete tumor regression without recurrence for about 3 months in 80 % of the mice population. Remarkably, this response was attained after a single treatment of 30-s duration with 3 W/cm^2 NIR with minimal local toxicity and no detectable systemic toxicity [76].

Scientists at Nanjing University and the Georgia Institute of Technology have employed the photoacoustic effect of carbon nanotubes (CNTs) to produce nanogrenades which can target and destroy cancer cells. Nanobombs are increasingly considered in the scientific community to selectively produce targeted microexplosions leading to the disruption of the unwanted cells in specific tissues. Irradiation with a Q-switched millisecond pulsed laser (1,064 nm) was used to generate the acoustic wave triggering a micro-explosions at tumor site (Fig. 19.5). This resulted in the death of 85 % of cells within 20 s with nanotube (folate targeted) uptake, while 90 % of cells without the nanotubes survived. Such a photoacoustic method for inducing cell death using carbon nanotubes can be proven to be superior to the photothermal method due to the fact that much less energy is required in the former. This in turn prevents excessive heating and negates chances of any non-targeted damage to the surrounding tissue [77, 78]. Such photothermal studies were also carried out by Zhou et al. in which SWCNTs were functionalized with PL-PEG-FA and PL-PEG-FITC and their in vitro and in vivo performance was studied using EMT6 cells and tumor-bearing mice respectively [66].

Infrared stimulation applied in the presence of nanotubes for approximately 10 s reduces cell survival to such low levels that it was comparable to cells incubated at

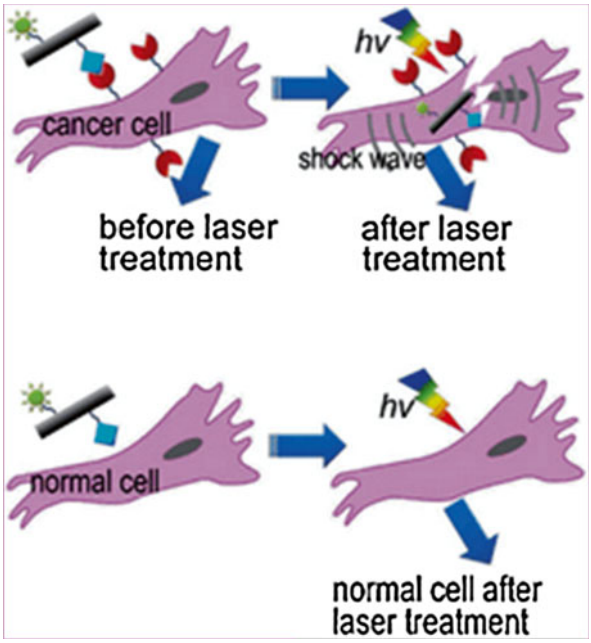

Fig. 19.5 Selective targeting and killing of cancer cells through photoacoustic effects. Modified and reprinted after permission from ref. [77]

42 °C for 2 h. Oxaliplatin uptake has also been demonstrated to increase after achieving rapid hyperthermia with nanotubes since the increase in temperature enhances cell permeability. This property could be advantageous in peritoneal dissemination of colorectal cancer. In such a case, nanotube solution can be injected intraperitoneally, and could be easily removed from the abdomen following hyperthermic chemotherapy by flushing the abdomen with saline. This approach has an excellent potential that can be explored for a rapid bench-to-bedside transition as a high impact clinical therapeutic agent in the surgical treatment of colorectal cancer since intravenously introduced nanotubes lack the ability to deliver therapeutic agents from the bloodstream to the peritoneum [70].

DNA-encased MWCNTs when irradiated with NIR resulted in a threefold reduction in the concentration of MWCNTs required to impart a 10 °C temperature increase in bulk solution temperature. Amount of heat generated increases with a linear dependence on irradiation time and laser power. A single intratumoral injection of MWCNTs (100 µl of a 500 µg/ml solution) followed by laser irradiation at 1,064 nm, 2.5 W/cm^2 completely eradicated PC3 xenograft tumors in 8/8 (100 %) of nude mice with no damage to normal tissue This was contrary to control that received only MWCNT injection or laser irradiation which exhibited growth rates indistinguishable from nontreated control tumors. Although no specific mechanism was proposed for enhanced heating due to DNA, the probable reason seems to be improved dispersibility as well as the extended chromophoricity imparted by DNA which enabled the large surface area of MWCNTs to interact with NIR [67]. Representative examples of photothermal therapy using CNTs are detailed in Table 19.3.

Table 19.3 Representative examples of photothermal therapy using CNTs (adapted and reprinted after permission from ref. [3])

Type of functionalization	Features of radiation	In vitro/in vivo system	Inference of study	Reference
A monoclonal antibody conjugated MWCNTs directed against CD133	NIR Laser $\lambda = 808$ nm; 600 J/cm^2	In vitro: adherent primary glioblastoma cells In vivo: murine flank tumor; primary glioblastoma cells	In vitro: specific internalization of targeted MWCNTs in primary clinical isolates of glioblastoma that expressed CD133, but not in cells which did not. In vivo: xenograft growth was abolished after NIR exposure in mice	[98]
Polyphenylene ethynylene (PPE) functionalized SWCNTs (π–π stacking)	Radiofrequency 0- to 2-kW, 13.56-MHz, Exposure for different times.	In vitro: HepG2 and Hep3B (hepatocellular cancer) and Panc-1 (pancreatic adenocarcinoma). In vivo: Rabbits bearing hepatic VX2 tumors (i.t inj.)	In vitro: dose and duration of exposure dependent cytotoxicity. In vivo: Complete tumor cell necrosis after 48 h	[74]
Phospholipid–PEG conjugate functionalized/ coated SWCNTs	NIR Laser $\lambda = 980$ nm; 450 J/cm^2	In vivo: murine flank tumor; EMT6 cells	In vivo: Treatment of murine breast cancer tumors with these modified SWCNTs reduced tumor growth and induced complete tumor regression in some mice following NIR exposure	[99, 100]
Pluronic F127 (1 % w/w) solubilized MWCNTs	NIR Nd:YAG laser (3 W/cm^2, $\lambda = 1{,}064$ nm), single 30-s treatment	In vitro: RENCA kidney cancer cells. In vivo: s.c implanted RENCA tumor bearing nude mice (intratumoral inj.)	In vitro: a 62-fold reduction in viability compared to control (MWCNT or laser alone). In vivo: No evident systemic toxicity, minimal local toxicity and. complete ablation of tumors and a >3.5-month durable remission in 80 % of mice (100 µg of MWCNT).	[76]
Oxidized MWCNTs in the presence of oxaliplatin	NIR laser, 3 W/cm^2, $\lambda = 1{,}064$ time: 8–11 s	In vitro: RKO and HCT 116 colorectal cancer cells	Increase in the temperature up to 42 °C caused enhanced uptake of drug leading to rapid death of cells	[70]
Gold plated SWCNTs conjugated to anti-human CD44 antibody	NIR laser, $\lambda = 850$ nm; 0.5 J/cm^2	In vivo: CD44+ expressing human breast cancer xenografts in mice	Photoacoustic detection of Rare CD44+ circulating cancer stem cells in the vasculature of nude mice bearing human breast cancer xenografts using a low powered laser along with ablation following extended irradiance with NIR	[101]
ssDNA encased MWCNTs	NIR laser, 2.5 W/cm^2, $\lambda = 1{,}064$ nm, Exposure time (30–70 s)	In vivo: PC3(prostrate cancer) xenograft tumor bearing mice, Single dose treatment intratumoral inj. of 50 µg	Complete and selective eradication of the tumor. Power and time dependent generation of heat by DNA-f-MWCNTs	[67]

In summary, the demonstration of photothermal transduction effect by nanotubes, has led to their exploration as photothermal agents employed in cancer killing by heating the nanotube to 50–70 °C through continuous laser irradiation at high power density (3.5–35 Wcm^2) for 3–4 min [77]. As photothermal effects result from continuous irradiation likewise photoacoustic effects materialize by employing a pulsed light source, causing transduction from light energy to acoustic pressure. Various studies conducted so far have reported the use of mainly SWCNTs for both photothermal and photoacoustic therapy. Fewer studies have been reported for MWCNTs relative to SWCNTs, in spite of the large chromophoric surface area displayed by MWCNTs owing to the presence of concentric tubes unlike the single tube present in SWCNTs. This warrants extensive comparative studies.

19.8 Carbon Nanotubes in Cancer Diagnostics and Imaging

The upsurge in the growth of diagnostic agents coupled with the integration of highly specific therapies has led to improvements in the outcome of therapeutic courses. So far, resection of the tumor at its early stage is the only effective cure for the disease, hence early screening and detection are of vital importance, but this is limited by the fact that most cancers are dormant during their early stage and do not reveal any overt signs. Conventional clinical cancer imaging modalities like X-ray, CT and MRI cannot detect subtle morphologic changes in the majority of early neoplastic disorders. Currently, positron emission tomography (PET) is a sensitive and accurate imaging technology available to identify early-stage alterations in molecular biology, even before there is any morphologic change, as it relies on changes in tissue biochemistry and metabolism [79–82].

Diagnosis of cancer in its early stages depends on the recognition of subtle changes in tissue properties such as, mechanical properties, optical absorption, and Rf absorption. A majority of diagnostic techniques translate such changes to give a detectable and visible output; for instance, a change in the ionic concentration affects the Rf absorption. Subsequent review sheds light on how nanotubes have been tuned to different diagnostic principles and used to develop new tools for early cancer detection. For a detailed account of the use of CNT in biosensing applications, the reader is referred to the review by Strano et al. [83].

CNT-based photoacoustic (PA) imaging is a novice modality offering higher spatial resolution and imaging of deeper tissues in comparison to most optical imaging techniques (e.g., absorbance and scattering of the emission light observed in fluorescence imaging). Principally, PA imaging is based on the fact that the laser pulses are absorbed by light-absorbing molecules, like endogenous molecules or contrast agents present in the biological milieu to generate heat and since most of the diseased tissue do not exhibit a natural photoacoustic contrast, there is no interference from tissue. The absorption of light induces transient thermoelastic expansion that leads to wideband ultrasonic emission, which can be traced by an ultrasound microphone to construct 2D or 3D images. Many nanoscale contrast agents for photoacoustic

imaging are currently available, which have strong absorbance in the NIR region, but suffer from the inability to target a diseased site in living subjects. As described in preceding sections, both MWCNTs and SWCNTs have been envisaged as photothermal agents due to their strong NIR absorbance. Intravenous administration of RGD (Arg-Gly-Asp) conjugated SWCNT-PL-PEG to target integrin $\alpha_v\beta_3$ receptors on tumor-bearing mice exhibited eight times greater photoacoustic signal in the tumor than mice injected with non-targeted nanotubes, which was corroborated further ex vivo using Raman microscopy [57]. SWCNTs have been widely used for photoacoustic imaging as they give maximal signal-to-noise ratio over other carbon materials like MWCNT, fullerenes, and graphitic microparticles, making them ideal agents in this arena of diagnostics, as has been demonstrated in in vivo models [84, 85].

Co-administering the contrast agents to enhance the photoacoustic signal seems to be a valid strategy in augmenting the signals from CNTs. Steering in a similar direction, indocyanine green (ICG) molecules have been loaded on PEGylated SWCNTs through pi–pi stacking. This increased the optical density of the nanoprobe about 20-fold at 780 nm which exhibited strong and separated absorbance spectra. Such a complex with tethered RGD had the ability to target cells and was shown to exhibit twentyfold better contrast than SWCNT without dye [86, 87]. The above findings suggest that targeted single-walled/multiwalled carbon nanotubes can be harnessed to image cancer tissue.

New diagnostic tools are often sought after to enable investigation of unstudied biological phenomena and improve clinical treatments. A reliable and early detection of cancer from biological fluids using novel technologies that possess sufficient spatial resolution based on the molecular signatures of cancer cell proteins via minimally invasive methods is still a high priority. Carbon nanotube field effect transistors (CNTFETs) are a novel class of diagnostic agents which are cost effective, require minimal sample volume, enable direct electrical readout, and have the ability to perform multiple detection of biomarkers based on Ag–Ab reactions between CNT conjugated Ab and cancer antigens [88].

A schematic representation of a prototype device structure of nanotube based sensor is demonstrated in Fig. 19.6. Device displaying p-type transistor behaviour with biomolecules immobilized on CNTs was produced using conducting channel made up of nanotubes, which connected the source and drain electrodes. IGF1R-(specific and nonspecific) mAbs when applied to such a device resulted in a decrease in the electrical conductance of the device. Application of BT474 breast cancer cells or MCF7 breast cancer cells to IGF1R-specific mAb-CNTFETs dramatically increased the conductance of the device. The density of IGF1R expressed on the surfaces of the breast cancer cells roughly correlated with the increase in conductance of the device.. Human BT474 breast cancer cells increased the conductance of the IGF1R-specific mAb–SWCNT devices by threefold, as compared to nanotube devices with nonspecific mAb. Greater IGF1R expressing Human MCF7 breast cancer cells increased the conductance by eightfold, which was not observed for R-cells lacking IGF1R [89]. Further, the surface charge affects the conductance of the SWCNTs, as the conductance of SWCNTs is very sensitive to surface charge

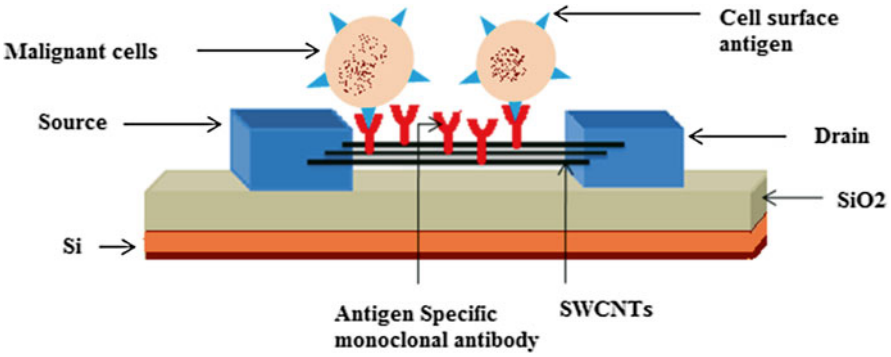

Fig. 19.6 Diagnostic electronic device based on carbon nanotube based transistors. Modified and reprinted after permission from ref. [89]

fluctuations and hence it has been observed that the charge transfer process leads to a change in conductance of the device during the interaction of antibodies and breast cancer cells mediated by the CNTs. Receptor-specific mAb represented specific nanoswitches which complete the circuit between the SWCNT and the cell surface receptors, leading to surge in the device conductance. Such modalities have tremendous potential as point of care devices in detecting circulating breast cancer cells in blood samples [90].

Prostate-specific antigen (PSA) is considered to be the best biomarker currently available for diagnosing and monitoring prostate cancer which is a glycoprotein consisting of 93 % peptide and 7 % sugar is produced exclusively by prostatic tissue [91]. The early stage detection of prostate cancer is the best hope for an increasing mortality rate, since there has been no curative therapy available [92]. Nanotransistors made from nanotubes spanning a gap between two metal contacts has been used by Yu et al. to demonstrated highly sensitive and selective electrochemical detection method for PSA. Compared to commercial immunoassays like ELISA which generally achieve detection limits from 10 to 100 pg mL^{-1} for many proteins in serum, such an approach had a detection limit of 4 pg mL^{-1}, for prostate specific antigen (PSA) with the least requirement of 10 μL of undiluted calf serum, and hence can be useful in clinical screening of cancer biomarkers and point-of-care diagnostics [69, 93].

Bareket et al. have developed a biosensor for the detection of formaldehyde based on coupling the selectivity of the enzyme formaldehyde dehydrogenase (FDH) with the highly efficient NADH detection system using a CNT-modified screen-printed electrode (SPE). Cancerous cells released formaldehyde when treated with formaldehyde based prodrugs. Based on the amperometric response to formaldehyde the signal was fivefold greater on the SPE/CNTs than on an unmodified SPE biosensor. This analytical tool for formaldehyde detection described herein can be useful to investigate the pharmacodynamics of formaldehyde-releasing prodrugs in cell cultures and bio-fluids [94].

Possessing distinct opto-electronic properties, carbon nanotubes are very sensitive to external stimuli, as a result the electronic structure of CNT is perturbed when they are subjected to molecular adsorption, which can be transduced into an optical signal either as a change in emission band intensity or wavelength, finding applications in a diagnostic space [95]. Different molecules interact differently with the nanotube surface depending on their spectroscopic signatures. If the degree of interaction between different analytes and the nanotubes is distinct, the former can be leveraged to concurrently perform multimodal analysis of chemotherapeutic drugs and reactive oxygen species or other biomarkers, with good sensitivity. The above concept has been exemplified by the real time detection and identification of such analytes within live 3T3 cells, the group demonstrated multiplexed optical detection from the first label-free nanoscale biosensor l to optically discriminate between genotoxins. Such a tool can be useful in prognosis as well as therapeutic monitoring to better control cancer therapy [96].

A group from Qingdao University have developed and reported an immunoassay for detection of AFP (alfa feto protein), a cancer biomarker based on signal amplification strategy using enzyme multilayers assembled multiwall carbon nanotubes (MWCNTs). The enhanced chemoluminescence system comprises luminol–H_2O_2-HRP (horseradish peroxidase)-BPB (bromophenol blue) utilizing MWCNTs as template carriers. The electrostatic layer-by-layer (LBL) self-assembly of negatively charged HRP and polycation PDDA onto MWCNT carriers achieved signal amplification with a detection limit of 8.0 pg/mL which is certainly more sensitive and two orders of magnitude lower than the standard ELISA method [97]. The above studies provide evidence that electrochemical methods utilizing CNT-based amplification strategies present many more advances over existing methods and are likely to influence the field of cancer diagnostics.

19.9 Conclusions and Prospective Remarks

Several technologies have made tremendous advancements in cancer research, but the perfect therapy is still a distant goal. Hence, the use of nanotechnology strategies to develop target-specific carriers which achieve better pharmacological profiles is gaining importance in the pharmaceutical and other industries. Nanohybrid materials are poised to emerge as a new avenue in cancer therapeutics. CNTs have not undergone the rigor of extensive investigations unlike the other nanocarriers, warranting more preclinical studies correlating the physical–chemical properties and biological response. The distinct properties of carbon nanotubes have made them a point of interest for many laboratories across the globe which have revealed improved biocompatibility and multimodal functionality to expect a better therapeutic outcome particularly for cancer therapy using CNTs. Cell internalization, higher drug loading capacity, photothermal response and electrochemically induced diagnostic capability are the critical features that make drug delivery systems multifunctional to be used effectively for cancer “theragnosis.”

References

1. Fabbro C, Ali-Boucetta H, Da Ros T, Kostarelos K, Bianco A, Prato M (2012) Targeting carbon nanotubes against cancer. Chem Commun 48:3911–3926
2. Ferrari M (2005) Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer 5:161–171
3. Thakare VS, Das M, Jain AK, Patil S, Jain S (2010) Carbon nanotubes in cancer theragnosis. Nanomedicine 5:1277–1301
4. Vashist SK, Zheng D, Pastorin G, Al-Rubeaan K, Luong JH, Sheu F-S (2011) Delivery of drugs and biomolecules using carbon nanotubes. Carbon 49:4077–4097
5. Joselevich E (2004) Electronic structure and chemical reactivity of carbon nanotubes: a chemist's view. Chemphyschem 5:619–624
6. Peigney A, Laurent C, Flahaut E, Bacsa R, Rousset A (2001) Specific surface area of carbon nanotubes and bundles of carbon nanotubes. Carbon 39:507–514
7. Ye Y, Ahn C, Witham C, Fultz B, Liu J, Rinzler A, Colbert D, Smith K, Smalley R (1999) Hydrogen adsorption and cohesive energy of single-walled carbon nanotubes. Appl Phys Lett 74:2307–2309
8. Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A (2006) Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol Sci 92:5–22
9. Thess A, Lee R, Nikolaev P, Dai H, Petit P, Robert J, Xu C, Lee YH, Kim SG, Rinzler AG (1996) Crystalline ropes of metallic carbon nanotubes. Science 273:483–487
10. Foldvari M, Bagonluri M (2008) Carbon nanotubes as functional excipients for nanomedicines: I. Pharmaceutical properties. Nanomedicine 4:173–182
11. Foldvari M, Bagonluri M (2008) Carbon nanotubes as functional excipients for nanomedicines: II. Drug delivery and biocompatibility issues. Nanomedicine 4:183–200
12. Lucente-Schultz RM, Moore VC, Leonard AD, Price BK, Kosynkin DV, Lu M, Partha R, Conyers JL, Tour JM (2009) Antioxidant single-walled carbon nanotubes. J Am Chem Soc 131:3934–3941
13. Jia N, Lian Q, Shen H, Wang C, Li X, Yang Z (2007) Intracellular delivery of quantum dots tagged antisense oligodeoxynucleotides by functionalized multiwalled carbon nanotubes. Nano Lett 7:2976–2980
14. Prakash S, Malhotra M, Shao W, Tomaro-Duchesneau C, Abbasi S (2011) Polymeric nanohybrids and functionalized carbon nanotubes as drug delivery carriers for cancer therapy. Adv Drug Deliv Rev 63:1340–1351
15. Niyogi S, Hamon M, Hu H, Zhao B, Bhowmik P, Sen R, Itkis M, Haddon R (2002) Chemistry of single-walled carbon nanotubes. Acc Chem Res 35:1105–1113
16. Jain AK, Dubey V, Mehra NK, Lodhi N, Nahar M, Mishra DK, Jain NK (2009) Carbohydrate-conjugated multiwalled carbon nanotubes: development and characterization. Nanomedicine 5:432–442
17. Tasis D, Tagmatarchis N, Georgakilas V, Gamboz C, Soranzo M-R, Prato M (2003) Supramolecular organized structures of fullerene-based materials and organic functionalization of carbon nanotubes. C R Chim 6:597–602
18. Ren Y, Pastorin G (2008) Incorporation of hexamethylmelamine inside capped carbon nanotubes. Adv Mater 20:2031–2036
19. Chen Z, Pierre D, He H, Tan S, Pham-Huy C, Hong H, Huang J (2011) Adsorption behavior of epirubicin hydrochloride on carboxylated carbon nanotubes. Int J Pharm 405:153–161
20. Tripisciano C, Kraemer K, Taylor A, Borowiak-Palen E (2009) Single-wall carbon nanotubes based anticancer drug delivery system. Chem Phys Lett 478:200–205
21. de Leon A, Jalbout AF, Basiuk VA (2008) SWNT–amino acid interactions: a theoretical study. Chem Phys Lett 457:185–190, http://dx.doi.org/10.1016/j.cplett.2008.03.079
22. Hilder TA, Hill JM (2008) Carbon nanotubes as drug delivery nanocapsules. Curr Appl Phys 8:258–261

23. Ma P-C, Zhang Y (2014) Perspectives of carbon nanotubes/polymer nanocomposites for wind blade materials. Renew Sustain Energy Rev 30:651–660
24. Yang D, Yang F, Hu J, Long J, Wang C, Fu D, Ni Q (2009) Hydrophilic multi-walled carbon nanotubes decorated with magnetite nanoparticles as lymphatic targeted drug delivery vehicles. Chem Commun:4447–4449. doi: 10.1039/b908012k
25. Gordon KB, Tajuddin A, Guitart J, Kuzel TM, Eramo LR, Vonroenn J (1995) Hand-foot syndrome associated with liposome-encapsulated doxorubicin therapy. Cancer 75:2169–2173
26. Gabizon A, Isacson R, Libson E, Kaufman B, Uziely B, Catane R, Ben-Dor CG, Rabello E, Cass Y, Peretz T (1994) Clinical studies of liposome-encapsulated doxorubicin. Acta Oncol 33:779–786
27. Ali-Boucetta H, Al-Jamal KT, McCarthy D, Prato M, Bianco A, Kostarelos K (2008) Multiwalled carbon nanotube–doxorubicin supramolecular complexes for cancer therapeutics. Chem Commun:459–461. doi: 10.1039/B712350G
28. Heister E, Neves V, Tîlmaciu C, Lipert K, Beltrán VS, Coley HM, Silva SRP, McFadden J (2009) Triple functionalisation of single-walled carbon nanotubes with doxorubicin, a monoclonal antibody, and a fluorescent marker for targeted cancer therapy. Carbon 47:2152–2160
29. Zhang X, Meng L, Lu Q, Fei Z, Dyson PJ (2009) Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. Biomaterials 30:6041–6047
30. Liu Z, Sun X, Nakayama-Ratchford N, Dai H (2007) Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery. ACS Nano 1:50–56
31. Chen M-L, He Y-J, Chen X-W, Wang J-H (2012) Quantum dots conjugated with Fe3O4-filled carbon nanotubes for cancer-targeted imaging and magnetically guided drug delivery. Langmuir 28:16469–16476
32. Wen S, Liu H, Cai H, Shen M, Shi X (2013) Drug delivery: targeted and ph-responsive delivery of doxorubicin to cancer cells using multifunctional dendrimer-modified multi-walled carbon nanotubes (Adv. Healthcare Mater. 9/2013). Adv Healthc Mater 2:1181. doi:10.1002/adhm.201370045
33. Modi CD, Patel SJ, Desai AB, Murthy R (2011) Functionalization and evaluation of PEGylated carbon nanotubes as novel drug delivery for methotrexate. J Appl Pharm Sci 1:103–108
34. Das M, Singh RP, Datir SR, Jain S (2013) Surface chemistry dependent "switch" regulates the trafficking and therapeutic performance of drug-loaded carbon nanotubes. Bioconjug Chem 24:626–639
35. Guan H, McGuire MJ, Li S, Brown KC (2008) Peptide-targeted polyglutamic acid doxorubicin conjugates for the treatment of αvβ6-positive cancers. Bioconjug Chem 19:1813–1821
36. Pastorin G, Wu W, Wieckowski S, Briand J-P, Kostarelos K, Prato M, Bianco A (2006) Double functionalisation of carbon nanotubes for multimodal drug delivery. Chem Commun:1182–1184. doi: 10.1039/B516309A
37. Samorì C, Ali-Boucetta H, Sainz R, Guo C, Toma FM, Fabbro C, da Ros T, Prato M, Kostarelos K, Bianco A (2010) Enhanced anticancer activity of multi-walled carbon nanotube–methotrexate conjugates using cleavable linkers. Chem Commun 46:1494–1496
38. Stanton RA, Gernert KM, Nettles JH, Aneja R (2011) Drugs that target dynamic microtubules: a new molecular perspective. Med Res Rev 31:443–481
39. Liu Z, Chen K, Davis C, Sherlock S, Cao Q, Chen X, Dai H (2008) Drug delivery with carbon nanotubes for in vivo cancer treatment. Cancer Res 68:6652–6660
40. Sobhani Z, Dinarvand R, Atyabi F, Ghahremani M, Adeli M (2011) Increased paclitaxel cytotoxicity against cancer cell lines using a novel functionalized carbon nanotube. Int J Nanomedicine 6:705–719
41. Yang F, Fu DL, Long J, Ni QX (2008) Magnetic lymphatic targeting drug delivery system using carbon nanotubes. Med Hypotheses 70:765–767
42. Oberoi HS, Nukolova NV, Kabanov AV, Bronich TK (2013) Nanocarriers for delivery of platinum anticancer drugs. Adv Drug Deliv Rev 65:1667–1685

43. Dhar S, Liu Z, Thomale J, Dai H, Lippard SJ (2008) Targeted single-wall carbon nanotube-mediated Pt (IV) prodrug delivery using folate as a homing device. J Am Chem Soc 130:11467–11476
44. Feazell RP, Nakayama-Ratchford N, Dai H, Lippard SJ (2007) Soluble single-walled carbon nanotubes as longboat delivery systems for platinum (IV) anticancer drug design. J Am Chem Soc 129:8438–8439
45. Wu W, Li R, Bian X, Zhu Z, Ding D, Li X, Jia Z, Jiang X, Hu Y (2009) Covalently combining carbon nanotubes with anticancer agent: preparation and antitumor activity. ACS Nano 3:2740–2750
46. Murakami T, Fan J, Yudasaka M, Iijima S, Shiba K (2006) Solubilization of single-wall carbon nanohorns using a PEG-doxorubicin conjugate. Mol Pharm 3:407–414
47. Weng X, Wang M, Ge J, Yu S, Liu B, Zhong J, Kong J (2009) Carbon nanotubes as a protein toxin transporter for selective HER2-positive breast cancer cell destruction. Mol Biosyst 5:1224–1231
48. Majoros IJ, Myc A, Thomas T, Mehta CB, Baker JR (2006) PAMAM dendrimer-based multifunctional conjugate for cancer therapy: synthesis, characterization, and functionality. Biomacromolecules 7:572–579
49. Shi X, Wang SH, Shen M, Antwerp ME, Chen X, Li C, Petersen EJ, Huang Q, Weber WJ Jr, Baker JR Jr (2009) Multifunctional dendrimer-modified multiwalled carbon nanotubes: synthesis, characterization, and in vitro cancer cell targeting and imaging. Biomacromolecules 10:1744–1750
50. Meng J, Duan J, Kong H, Li L, Wang C, Xie S, Chen S, Gu N, Xu H, Yang XD (2008) Carbon nanotubes conjugated to tumor lysate protein enhance the efficacy of an antitumor immunotherapy. Small 4:1364–1370
51. Xiao Y, Gao X, Taratula O, Treado S, Urbas A, Holbrook RD, Cavicchi RE, Avedisian CT, Mitra S, Savla R (2009) Anti-HER2 IgY antibody-functionalized single-walled carbon nanotubes for detection and selective destruction of breast cancer cells. BMC Cancer 9:351
52. Villa CH, Dao T, Ahearn I, Fehrenbacher N, Casey E, Rey DA, Korontsvit T, Zakhaleva V, Batt CA, Philips MR (2011) Single-walled carbon nanotubes deliver peptide antigen into dendritic cells and enhance IgG responses to tumor-associated antigens. ACS Nano 5:5300–5311
53. Bhirde AA, Patel V, Gavard J, Zhang G, Sousa AA, Masedunskas A, Leapman RD, Weigert R, Gutkind JS, Rusling JF (2009) Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. ACS Nano 3:307–316
54. McDevitt MR, Chattopadhyay D, Kappel BJ, Jaggi JS, Schiffman SR, Antczak C, Njardarson JT, Brentjens R, Scheinberg DA (2007) Tumor targeting with antibody-functionalized, radiolabeled carbon nanotubes. J Nucl Med 48:1180–1189
55. Chakravarty P, Marches R, Zimmerman NS, Swafford AD-E, Bajaj P, Musselman IH, Pantano P, Draper RK, Vitetta ES (2008) Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes. Proc Natl Acad Sci 105:8697–8702
56. Kam NWS, O'Connell M, Wisdom JA, Dai H (2005) Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. Proc Natl Acad Sci U S A 102:11600–11605
57. Liu Z, Cai W, He L, Nakayama N, Chen K, Sun X, Chen X, Dai H (2007) In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. Nat Nanotechnol 2:47–52
58. Ou Z, Wu B, Xing D, Zhou F, Wang H, Tang Y (2009) Functional single-walled carbon nanotubes based on an integrin αvβ3 monoclonal antibody for highly efficient cancer cell targeting. Nanotechnology 20:105102
59. Seow Y, Wood MJ (2009) Biological gene delivery vehicles: beyond viral vectors. Mol Ther 17:767–777
60. Gao K, Huang L (2008) Nonviral methods for siRNA delivery. Mol Pharm 6:651–658
61. El-Aneed A (2004) Current strategies in cancer gene therapy. Eur J Pharmacol 498:1–8

62. Albertorio F, Hughes ME, Golovchenko JA, Branton D (2009) Base dependent DNA–carbon nanotube interactions: activation enthalpies and assembly–disassembly control. Nanotechnology 20:395101
63. Krajcik R, Jung A, Hirsch A, Neuhuber W, Zolk O (2008) Functionalization of carbon nanotubes enables non-covalent binding and intracellular delivery of small interfering RNA for efficient knock-down of genes. Biochem Biophys Res Commun 369:595–602
64. Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand JP, Prato M, Kostarelos K, Bianco A (2004) Functionalized carbon nanotubes for plasmid DNA gene delivery. Angew Chem 116:5354–5358
65. Zhang Z, Yang X, Zhang Y, Zeng B, Wang S, Zhu T, Roden RB, Chen Y, Yang R (2006) Delivery of telomerase reverse transcriptase small interfering RNA in complex with positively charged single-walled carbon nanotubes suppresses tumor growth. Clin Cancer Res 12:4933–4939
66. Zhou F, Resasco DE, Chen WR, Xing D, Ou Z, Wu B (2009) Cancer photothermal therapy in the near-infrared region by using single-walled carbon nanotubes. J Biomed Opt 14:021009
67. Ghosh S, Dutta S, Gomes E, Carroll D, D'Agostino R Jr, Olson J, Guthold M, Gmeiner WH (2009) Increased heating efficiency and selective thermal ablation of malignant tissue with DNA-encased multiwalled carbon nanotubes. ACS Nano 3:2667–2673
68. König K (2000) Multiphoton microscopy in life sciences. J Microsc 200:83–104
69. Weissleder R (2001) A clearer vision for in vivo imaging. Nat Biotechnol 19:316–317
70. Levi-Polyachenko NH, Merkel EJ, Jones BT, Carroll DL, Stewart JH IV (2009) Rapid photothermal intracellular drug delivery using multiwalled carbon nanotubes. Mol Pharm 6:1092–1099
71. Kim P, Odom TW, Huang J-L, Lieber CM (1999) Electronic density of states of atomically resolved single-walled carbon nanotubes: Van Hove singularities and end states. Phys Rev Lett 82:1225
72. Bachilo SM, Strano MS, Kittrell C, Hauge RH, Smalley RE, Weisman RB (2002) Structure-assigned optical spectra of single-walled carbon nanotubes. Science 298:2361–2366
73. Govorov AO, Richardson HH (2007) Generating heat with metal nanoparticles. Nano Today 2:30–38
74. Gannon CJ, Cherukuri P, Yakobson BI, Cognet L, Kanzius JS, Kittrell C, Weisman RB, Pasquali M, Schmidt HK, Smalley RE (2007) Carbon nanotube-enhanced thermal destruction of cancer cells in a noninvasive radiofrequency field. Cancer 110:2654–2665
75. Torti SV, Byrne F, Whelan O, Levi N, Ucer B, Schmid M, Torti FM, Akman S, Liu J, Ajayan PM (2007) Thermal ablation therapeutics based on CNx multi-walled nanotubes. Int J Nanomedicine 2:707
76. Burke A, Ding X, Singh R, Kraft RA, Levi-Polyachenko N, Rylander MN, Szot C, Buchanan C, Whitney J, Fisher J (2009) Long-term survival following a single treatment of kidney tumors with multiwalled carbon nanotubes and near-infrared radiation. Proc Natl Acad Sci 106:12897–12902
77. Kang B, Yu D, Dai Y, Chang S, Chen D, Ding Y (2009) Cancer-cell targeting and photoacoustic therapy using carbon nanotubes as "Bomb" agents. Small 5:1292–1301
78. Hecht D (2009) "Nanobombs" shock cancer cells: nanomedicine. Mater Today 12:8, http://dx.doi.org/10.1016/S1369-7021(09)70099-9
79. Singh R, Pantarotto D, McCarthy D, Chaloin O, Hoebeke J, Partidos CD, Briand J-P, Prato M, Bianco A, Kostarelos K (2005) Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: toward the construction of nanotube-based gene delivery vectors. J Am Chem Soc 127:4388–4396
80. Manne U, Srivastava R-G, Srivastava S (2005) Keynote review: recent advances in biomarkers for cancer diagnosis and treatment. Drug Discov Today 10:965–976
81. Xue Y, Bao L, Xiao X, Ding L, Lei J, Ju H (2011) Noncovalent functionalization of carbon nanotubes with lectin for label-free dynamic monitoring of cell-surface glycan expression. Anal Biochem 410:92–97

82. Ananta JS, Matson ML, Tang AM, Mandal T, Lin S, Wong K, Wong ST, Wilson LJ (2009) Single-walled carbon nanotube materials as T 2-weighted MRI contrast agents. J Phys Chem C 113:19369–19372
83. Kruss S, Hilmer AJ, Zhang J, Reuel NF, Mu B, Strano MS (2013) Carbon nanotubes as optical biomedical sensors. Adv Drug Deliv Rev 65:1933–1950
84. Pramanik M, Song KH, Swierczewska M, Green D, Sitharaman B, Wang LV (2009) In vivo carbon nanotube-enhanced non-invasive photoacoustic mapping of the sentinel lymph node. Phys Med Biol 54:3291
85. Pramanik M, Swierczewska M, Wang LV, Green D, Sitharaman B (2009) Single-walled carbon nanotubes as a multimodal-thermoacoustic and photoacoustic-contrast agent. J Biomed Opt 14:034018
86. Adl Z, Liu Z, Bodapati S, Teed R, Vaithilingam S, Khuri-Yakub BT, Chen X, Dai H, Gambhir SS (2010) Ultrahigh sensitivity carbon nanotube agents for photoacoustic molecular imaging in living mice. Nano Lett 10:2168–2172
87. de la Zerda A, Bodapati S, Teed R, May SY, Tabakman SM, Liu Z, Khuri-Yakub BT, Chen X, Dai H, Gambhir SS (2012) Family of enhanced photoacoustic imaging agents for high-sensitivity and multiplexing studies in living mice. ACS Nano 6:4694–4701
88. Maehashi K, Katsura T, Kerman K, Takamura Y, Matsumoto K, Tamiya E (2007) Label-free protein biosensor based on aptamer-modified carbon nanotube field-effect transistors. Anal Chem 79:782–787
89. Panchapakesan B, Cesarone G, Liu S, Teker K, Wickstrom E (2005) Single-wall carbon nanotubes with adsorbed antibodies detect live breast cancer cells. Nanobiotechnology 1:353–360
90. Teker K (2008) Bioconjugated carbon nanotubes for targeting cancer biomarkers. Mater Sci Eng B 153:83–87
91. Loeb S, Catalona WJ (2007) Prostate-specific antigen in clinical practice. Cancer Lett 249:30–39
92. Kim JP, Lee BY, Lee J, Hong S, Sim SJ (2009) Enhancement of sensitivity and specificity by surface modification of carbon nanotubes in diagnosis of prostate cancer based on carbon nanotube field effect transistors. Biosens Bioelectron 24:3372–3378
93. Yu X, Munge B, Patel V, Jensen G, Bhirde A, Gong JD, Kim SN, Gillespie J, Gutkind JS, Papadimitrakopoulos F (2006) Carbon nanotube amplification strategies for highly sensitive immunodetection of cancer biomarkers. J Am Chem Soc 128:11199–11205
94. Bareket L, Rephaeli A, Berkovitch G, Nudelman A, Rishpon J (2010) Carbon nanotubes based electrochemical biosensor for detection of formaldehyde released from a cancer cell line treated with formaldehyde-releasing anticancer prodrugs. Bioelectrochemistry 77:94–99
95. Dukovic G, White BE, Zhou Z, Wang F, Jockusch S, Steigerwald ML, Heinz TF, Friesner RA, Turro NJ, Brus LE (2004) Reversible surface oxidation and efficient luminescence quenching in semiconductor single-wall carbon nanotubes. J Am Chem Soc 126: 15269–15276
96. Heller DA, Jin H, Martinez BM, Patel D, Miller BM, Yeung T-K, Jena PV, Höbartner C, Ha T, Silverman SK (2009) Multimodal optical sensing and analyte specificity using single-walled carbon nanotubes. Nat Nanotechnol 4:114–120
97. Bi S, Zhou H, Zhang S (2009) Multilayers enzyme-coated carbon nanotubes as biolabel for ultrasensitive chemiluminescence immunoassay of cancer biomarker. Biosens Bioelectron 24:2961–2966
98. Wang C-H, Chiou S-H, Chou C-P, Chen Y-C, Huang Y-J, Peng C-A (2011) Photothermolysis of glioblastoma stem-like cells targeted by carbon nanotubes conjugated with CD133 monoclonal antibody. Nanomedicine 7:69–79

99. Zhou F, Xing D, Wu B, Wu S, Ou Z, Chen WR (2010) New insights of transmembranal mechanism and subcellular localization of noncovalently modified single-walled carbon nanotubes. Nano Lett 10:1677–1681
100. Zhou F, Wu S, Wu B, Chen WR, Xing D (2011) Mitochondria-targeting single-walled carbon nanotubes for cancer photothermal therapy. Small 7:2727–2735
101. Galanzha EI, Kim JW, Zharov VP (2009) Nanotechnology-based molecular photoacoustic and photothermal flow cytometry platform for in-vivo detection and killing of circulating cancer stem cells. J Biophotonics 2:725–735

Part VII
Advanced Characterization Techniques for Nanocarriers

Chapter 20
Physical and Biophysical Characteristics of Nanoparticles: Potential Impact on Targeted Drug Delivery

Chiranjeevi Peetla and Vinod Labhasetwar

Abbreviations

DMAB	Didodecyldimethylammonium bromide
DMAB	Didodecyldimethylammonium bromide
NPs	Nanoparticles
PEG	Poly(ethylene glycol)
PLA	Polylactide
PLGA	Polylactide-*co*-glycolide
RES	Reticuloendothelial system
TAB	Cetyltrimethylammonium bromide
TAT	*Trans*-activating transcriptor

20.1 Introduction

In nanomedicine, targeting refers to directing therapeutics to pathological sites using nanocarriers as a delivery mechanism [1]. Nanotechnology-based targeted therapies are promising because (a) they potentially offer effective treatment at lower doses of therapeutics, thereby avoiding or reducing side effects, and (b) with

C. Peetla
Department of Biomedical Engineering, Lerner Research Institute, Cleveland Clinic, Cleveland, OH USA

V. Labhasetwar, Ph.D. (✉)
Department of Biomedical Engineering/ND20, Lerner Research Institute and Taussig Cancer Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA
e-mail: labhasv@ccf.org

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_20

sustained-release nanoparticles (NPs), therapeutic drug levels can be maintained for a prolonged period, thus minimizing the frequency of dosing, improving efficacy and patients' compliance with the treatment [2, 3]. Here, for simplicity's sake, we have categorized different types of nanocarriers used for drug delivery as NPs. First-generation NPs demonstrated promising results in achieving safe and effective treatment compared with conventional chemotherapeutic agents in clinical settings. The efficacy of NPs has primarily been attributed to their ability to deliver higher doses of drugs than drugs alone without increasing toxicity. This ability was particularly noticeable with anticancer drugs, whose use must be carefully limited due to their increased toxicity at doses high enough to achieve tumor regression or control metastatic progression. For instance, the FDA-approved anticancer nanomedicines Doxil® (a liposomal formulation of doxorubicin) and Abraxane® (a nanoscale albumin-bound form of paclitaxel) have been administered via NPs with significantly reduced side effects to patients [4–6]. Second-generation NPs are aimed at achieving precise delivery of therapeutics to the targeted tissues or cells. It is expected that the amount of drug required to achieve therapeutic effects with second-generation NPs may be quite low, thus eventually reducing side effects and providing better efficacy. In addition, intracellular drug delivery can overcome the most critical limitation of drug actions, i.e., multidrug resistance, which has been a major hurdle in cancer chemotherapy [7].

NP targeting strategies can be broadly classified into two categories: passive and active targeting [8]. Passive targeting depends on physicochemical properties of NPs and disease pathology, which may preferentially aid in the accumulation of NPs in the target tissue and hence reduce nonspecificity [9]. For instance, passive targeting of NPs can be achieved in tumors and inflamed tissues [10]. In tumors, vascular leakiness is the result of increased angiogenesis [11]; therefore, NP accumulation in tumor tissue is relatively higher than in normal tissues, a condition commonly known as the enhanced permeation retention or EPR effect. Similarly, under inflammatory conditions, there is increased vascular/tissue permeability, such as in arthritic joints, due to an excessive amount of inflammatory cytokines produced [12]. In active targeting, NP delivery is mediated through targeting ligands such as antibodies, peptides, and so on. This strategy aims at intracellular delivery of NPs due to specific interactions of the targeting moiety with a cell-surface antigen or receptor [13–16]. NPs are coupled to one or multiple ligands that facilitate several drug molecules encapsulated in NPs to be imported into the target cell population by means of receptor–ligand interaction.

In general, in the passive targeting strategy, physical properties of NPs such as size, shape, surface charge, hydrophilicity, hydrophobicity are modified, whereas in active targeting, NPs are conjugated to ligands for receptors that are overexpressed in diseased cells or tissue. Currently approved NP formulations are made without any targeting ligand; however, 13 ligand-targeted formulations, primarily conjugated to antibodies and developed for cancer therapy, have progressed to Phase I/II clinical trials. Although ligand-targeted NPs have been proven safe and efficacious in preclinical models, their efficacy in clinical studies has not been unambiguously proven yet [17].

One emerging approach entails developing targeted NPs without using targeting ligands. This method is based on understanding how physical and biophysical properties of NPs influence their interactions with biomolecules (such as proteins and the cell membrane), cells, and tissues, and the biodistribution, targeting, and clearance of these NPs from the body. Such an approach can potentially avoid complex conjugation chemistry with targeting ligands. Such biochemical constructs have, in part, limited the translation of potential therapies into clinical practice because of the challenges presented in manufacturing them, as well as in confirming their reproducibility, managing their high cost, and complying with additional regulatory requirements [18].

20.2 Impact of Physical Characteristics of NPs on Cellular Uptake, Biodistribution, and Targeting

Targeting approaches can be developed by understanding the effects of physical and/or chemical characteristics of NPs, such as size, geometry/shape, surface charge, surface chemistry, hydrophilicity/hydrophobicity, roughness, rigidity, composition on cellular uptake, biodistribution, and hence ease of targeting.

20.2.1 Role of NP Size in Cellular Uptake and Targeting

The effects of NP size (diameter) on targeting ability have been extensively investigated with spherical NPs. It has been shown that NP size affects protein adsorption, blood circulation half-life, and renal clearance, as well as their ability to deliver therapeutics to the target site. For instance, Choi et al. [19] showed that only NPs below 5.5 nm in diameter clear from the blood circulation via the kidneys. As their size increases, NPs usually show a greater propensity to accumulate in the liver and spleen. No one particular NP size suits all clinical applications; however, generally for in vivo applications, NPs are prepared in the size range 10–300 nm.

Toy et al. [20] showed that tumor blood flow influences the localization of intravenously injected liposomes within the tumor in a size-dependent manner. The studies demonstrated that large liposomes (100 nm) effectively extravasate in fast-flow regions of the tumor, whereas smaller liposomes (65 nm) localized better in slow-flow regions. The study also showed that retention of actively targeted liposomes within the tumor varies with liposome size. In this case, liposomes of 100 nm showed no benefits at any flow rate, but active targeting substantially increased deposition of 30-nm liposomes in slow-flow tumor regions (∼12-fold increase) compared with nontargeted 30-nm liposomes. The results of these studies clearly demonstrate that not only the characteristics of NPs but also the biological environment around the disease condition influences targeting of NPs.

Reddy et al. [21] showed that by controlling the size, NPs can be delivered to antigen-presenting cells, specifically dendritic cells, following their interstitial injection. In this study, the authors investigated the delivery of poly(ethylene glycol) (PEG)- stabilized poly(propylene sulfide) NPs (of 20, 45, and 100 nm diameter) to dendritic cells in the lymph nodes. The results demonstrated that, compared to 100-nm NPs, NPs of 20 and 45 nm were effectively retained by lymph nodes for at least 5 days, without requiring the use of any specific targeting ligand. The small NPs were shown to have internalized exclusively by nodal resident dendritic cells and other antigen-presenting cells. In a separate study, Manolova et al. [22] showed that NPs are drawn toward the draining lymph node in a size-dependent manner. For comparison, polystyrene fluorescent NPs of different sizes (20–1,000 nm) were injected into the footpads of mice. The authors showed that large NPs (>500 nm) remained associated mostly with dendritic cells at the injection site, whereas small NPs (20–200 nm) were found in lymph node-resident dendritic cells and macrophages, suggesting free drainage of small NPs to the lymph nodes [22]. Thus the optimal size of NPs may depend on the target tissue or cell population or the route of administration.

A recent study aimed at determining the optimal size for maximum NP uptake by various tumor cell types suggested that even a 10-nm deviation from the optimal size can significantly affect NP uptake [23]. In an in vivo study, Schadlich et al. [24] showed the significance of tumor vascularity and the effect of narrow NP size distributions on targeting efficiency. Furthermore, the study investigated the size-dependent accumulation of fluorescently labeled polylactide (PLA)-PEG polymeric NPs using two different tumor xenograft models: HT20 colon and A2780 ovarian carcinoma. These two models showed quite different tumor structures, growth rates, and microenvironments. Using an in vivo fluorescence imaging technique, the pattern of accumulation of PLA-PEG NPs was found to be significantly different in both tumor types; fluorescence was observed primarily from the core region in the case of the HT29 tumor but not the A2780 tumors [24]. The results thus clearly suggest that NP accumulation in tumors depends on the tumor type, which can also affect the efficacy of the NP-mediated drug therapy.

We evaluated the effect of heterogeneity of NPs in two formulations. Most NP formulations comprise different NP populations that have distinctly different physical properties. To mimic this situation, we monitored the biodistribution of two different sized polylactide-*co*-glycolide (PLGA)-NPs (small and large, mean diameter = 70 nm and 202 nm, respectively), formulated using the same composition of polymer and emulsifier, mixed at a 1:1 w/w ratio and injected intravenously in a prostate tumor xenograft mouse model. Two formulations were labeled with two different near-infrared dyes with emission wavelengths that can easily be distinguished from each other. This method allowed us to monitor biodistribution of small and large PLGA-NPs simultaneously. The results demonstrated notably different biodistributions; small NPs showed longer circulation time than large NPs ($t_{1/2}$ = 96 min vs. 13 min), accounted for 75 % of total NPs accumulated in the tumor, and showed a 13-fold greater tumor-to-liver signal intensity ratio than large

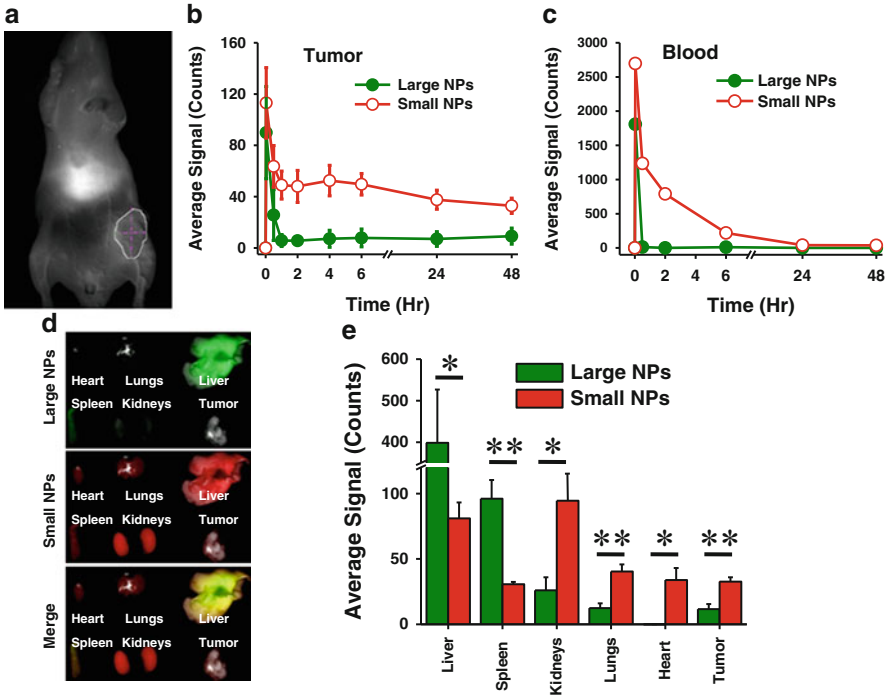

Fig. 20.1 *Quantification of* in vivo *signal of small and large NPs.* Equal doses of large and small NPs loaded with different amounts of near-infrared dye were mixed and injected intravenously in prostate tumor-bearing mice. (**a**) Region of interest (ROI) created over the anatomic location of tumor. (**b**) Tumor accumulation of NPs. IV injection results in rapid uptake of NPs, which gradually drain out of tumor. $p = 0.03$ at 4 h post injection in tumor. Data are shown as mean ± s.e.m, $n = 6$. (**c**) Clearance of large and small NPs from blood. (**d**) Ex vivo imaging of tissues excised from mouse 48 h post intravenous injection of large and small NPs. Mice were perfused with heparinized saline to remove NPs remaining in blood vessels before ex vivo imaging. *Red* indicates small NPs and *green* large NPs; white indicates autofluorescence. Large NPs show a greater uptake into organs of the RES but little accumulation in other tissues. Small NPs show a greater uptake into tumor, kidneys, lungs, and heart. (**e**) Average signals from excised tissue after imaging with Maestro Ex were determined by drawing ROIs around each tissue. Large NPs showed a fivefold accumulation in liver compared with small NPs. Small NPs showed a threefold accumulation in tumor with increased uptake by kidney. $*p \leq 0.03$, $**p \leq 0.01$. Data are shown as mean ± s.e.m, $n = 6$. Reproduced from ref. [25]

NPs [25] (Fig. 20.1). The data thus underscore the importance of formulating NPs with specific properties to enhance their targeting efficacy and minimize nonspecific distribution.

At the cellular level, NP size has been shown to affect the rate of cellular uptake of NPs, as it influences the mechanisms of internalization. Phagocytosis and pinocytosis (or fluid-phase uptake) are two major endocytic mechanisms by which cells take up NPs [26]. Large NPs (>1 μm diameter) are generally internalized by

phagocytotic mechanisms [27]. Pinocytosis is more relevant to NP cellular uptake and can occur either via adsorptive pinocytosis or via receptor-mediated endocytosis [28]. It appears that NPs <50 nm accumulate in lymphatics and NPs of 70–200 nm accumulate effectively within tumor tissue and that the upper limit for extravasation into solid tumors is ~400 nm [29].

NPs of only one size are not optimal for targeting different tumor types, and the extent of a tumor's vascularity must also be taken into account. In addition, the optimal size of NPs depends on the cells/tissue of interest and the route of administration. Thus it can be stated that one standard formulation of NPs cannot be optimal for all disease conditions.

20.2.2 Role of NP Geometry in Cellular Uptake and Targeting

Champion et al. [30] have shown the ability to form nanoparticles and microparticles of different shapes using two different techniques (Fig. 20.2). Several studies have reported that NP geometry affects the cellular uptake, blood-circulation time (due to differences in adhesion to blood vessels), and biodistribution of NPs [31]. For instance, Decuzzi et al. [32] reported that spherical and nonspherical NPs have varying distributions following intravenous injection. This study also found that compared with rod-shaped and spherical NPs, discoidal NPs showed less accumulation in the liver. Kolhar et al. [33] demonstrated that the specificity of endothelial targeting can be enhanced by engineering the shape of ligand-displaying NPs. Using in vitro microfluidic systems that mimic the vasculature, the study predicted that compared with their spherical counterparts, rod-shaped NPs would show greater

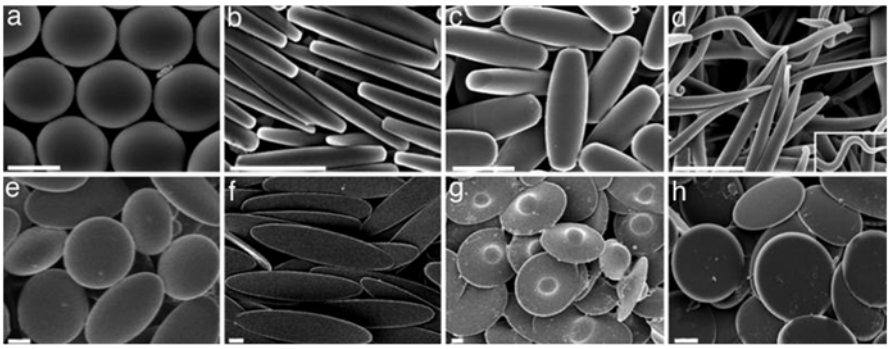

Fig. 20.2 *Nanoparticles and microparticles with different shapes.* Two different methods were developed to make nanoparticles and microparticles of different shapes. Micrographs of shapes made by one of the following techniques: (**a**) spheres; (**b**) rectangular disks; (**c**) rods; (**d**) worms; (**e**) oblate ellipses; (**f**) elliptical disks; (**g**) shapes like "unidentified flying objects"; and (**h**) circular disks. (Scale bars: 2 μm). The group has extensively studied the effect of shape on cellular uptake and biodistribution (reproduced from ref. [30]

accumulation in vascular endothelium. Based on a mathematical modeling of NP–surface interaction, the group has attributed higher specificity of nanorods to endothelial cells due to their favored adhesion and reduced shear-induced detachment than spherical NPs [34, 35]. In another study, Chauhan et al. [36] demonstrated that rod-shaped NPs are transported through porous media more rapidly than spherical NPs of the same hydrodynamic size (34–36 nm). This group also revealed that nanorods diffuse more quickly (by up to an order of magnitude) than nanospheres through pores similar in size (100–400 nm) to those of tumor vasculature. The follow-up in vivo study demonstrated that nanorods penetrate tumors 4.1 times as rapidly as nanospheres.

At the cellular level, adhesion strength is lower for spherical NPs, but their internalization rate is higher than for nanorods. Studies have also shown that among NPs of either rod or sphere design, spherical NPs are taken up by cells more readily, whereas disk-like, cylindrical, and hemispherical NPs are more efficient than spherical ones at evading phagocytic cellular uptake [37]. Shape, volume, contact area, local curvature of NPs at the contact point, and initial orientation of NPs are all critical to the nature of the interaction between NPs and the lipid bilayer [38]. DeSimone et al. [39] found that, in contrast to cylindrical NPs, the internalization of rod-like NPs with high aspect ratios (depth: 150 nm, height: 450 nm, and volume: 0.00795 μm) occurs more rapidly in HeLa cells, regardless of NP volume. In an interesting study, Peiris et al. [40] demonstrated that chain-shaped NPs (comprising four iron oxide nanospheres chemically linked in a linear assembly) target metastatic tumors better than spherical NPs do. Using multimodal in vivo imaging, the authors showed that the chain configuration of NPs enhances multivalent docking to the $\alpha_v\beta_3$ integrin receptors, which are overexpressed on tumor vasculature. Discher's group [41] has extensively studied the effects of NP shape. They have shown that flexible, worm-like filomicelles increase the amount of drug delivered to tumors and shrink tumors more effectively than spherical micelles do [41]. All these examples clearly demonstrate the role that NP shape plays in cellular uptake, biodistribution, and targeting of NPs. However, the shape effect in vitro and in vivo may differ and could very much depend upon cell type and target organ.

20.2.3 Role of NP Surface Charge in Cellular Uptake and Targeting

The surface charge of NPs has been shown to influence their cell-entry, circulation half-life, and tissue retention capabilities. Cationic NPs are known to have short half-lives in vivo due to their interactions with blood proteins; this results in complement activation and recognition of injected NPs by circulating monocytes and rapid clearance via the reticuloendothelial system (RES) [28]. It is believed that at the cellular level, compared with anionic NPs, cationic NPs show greater cellular uptake in the absence of serum in the media; this effect has been attributed to their

electrostatic interaction with the cell membrane. Cationic surfaces on NPs can interact with the negatively charged phospholipid head groups, proteins, and glycans on the cell surface, therefore NPs with cationic surfaces promote cellular binding, resulting in either uptake through endocytosis or direct penetration through the cell-surface membrane [27, 42]. Highly cationic NP surfaces could also bypass endocytic modes of entry, as the NPs can enter cells by creating holes in the cellular bilayer, but that might also cause cytotoxicity due to the diffusion of cytoplasmic content and interactions with intracellular components [27]. Anionic NPs show better cellular uptake than neutral NPs [43]. NPs with neutral methoxy surface groups have been found to be more immunocompatible than NPs with carboxy or amino surface groups [44]. The immunocompatibility properties of lipid-polymeric NPs with different surface charges were investigated by measuring the effects of carboxy, amino, and methoxy terminated PEGylated NPs on the degree of complement system activation, human plasma protein binding, and coagulation system activation. The study demonstrated that neutral methoxy surface groups are more immunocompatible than NPs with anionic or cationic surface charges [44]. In general, NPs with a surface charge of <15 mV demonstrate minimal macrophage uptake, and hence longer circulation times and greater retention once in tumor tissue [45]. Cationic NPs show improved cellular uptake but cause nonspecific interactions in vivo [46]. It would be ideal to design NPs that at first would carry a negative or neutral surface charge for a longer circulation half-life but which could then reverse their surface charge to cationic at the site of action to facilitate uptake of NPs into the targeted cell [47].

Understanding the effects of even one physical parameter on biodistribution is quite complex because that parameter also simultaneously changes other characteristics of NPs. For instance, in our recent study, we demonstrated that PLGA-NPs of different sizes also show different surface charges. Although both small and large NPs were prepared using the same composition of polymer and emulsifier, each acquired a different zeta potential. Small NPs were less negative (almost neutral [$\zeta = -0.81$ mV]) than large NPs ($\zeta = -16.15$ mV) [25]. In summary, investigators have achieved a general understanding of the effects of the surface charge of NPs on their cellular uptake, interactions with proteins, immunocompatibility, and circulation half-life.

20.2.4 Role of Hydrophilicity and Hydrophobicity in Cellular Uptake and Targeting

Surface hydrophobicity influences protein adsorption and cellular uptake of NPs, hence their ability to deliver therapeutics to targeted sites. Normally, NPs that are more hydrophobic than the cell-surface membrane are easily taken up by cells [27]; however, opsonins, which cause NP blood clearance by cells of the immune system [48],

also have a high affinity for hydrophobic surfaces [49]. To prevent unwanted protein binding from plasma, NP surfaces are coated with PEG, a highly hydrophilic polymer. PEG coating is known to prolong the circulation half-life of NPs [50]. In addition, the density and configuration of PEG on the NP surface has been found to affect opsonization and biodistribution of NPs [51]. PEG configurations on the surfaces of NPs can exist as either extended brush-like structures or coiled mushroom or mushroom/brush intermediates. Of these two configurations, predominant brush-like PEG surfaces have been shown to sterically suppress binding of opsonins such as the C3 protein. In addition, surface effects (such as nanoscale "roughness") have been shown to minimize repulsive forces between cells and NP surfaces [52, 53]. However, several studies have recently reported opsonization and uptake of certain PEGylated NPs by the RES, indicating that PEGylation is not entirely effective in preventing protein adsorption [54]. It is possible that desorption of PEG or displacement of PEG with proteins may occur over time [55, 56].

20.3 Impact of NP Interfacial Interactions on Cellular Uptake, Biodistribution, and Targeting

By virtue of advances in material and colloidal sciences, NPs can be prepared with different size, shape, surface property, drug loading, and release features. However, targeted drug therapy remains a challenge. This may in part be due to the gap between the in vitro methods used for NP characterization and their relevance to in vivo conditions. The in vitro models used do not provide information about biophysical responses of cells upon contact with NPs, which may be involved in the cellular uptake process. For example, the NP uptake process may include NP binding with the cell membrane, conformational changes in membrane proteins, and changes in local membrane lipid arrangement [57]. Basically, current characterization techniques do not account for interactions occurring at the interface between NPs and biological systems. In particular, we have very limited knowledge about the role of dynamic interactions between NP and proteins or between NP and cell membrane lipids and their relevance to the biodistribution and targeting of NPs.

20.3.1 Biophysical Characterization of NP–Protein Interactions for Targeting Tissues

Following intravenous injection, the NP surface is covered by a protein corona. The adsorbed protein corona can alter the NP's size, surface charge, aggregation state, and other characteristics. Furthermore, the NP–protein interface undergoes continuous changes because of the dynamic nature of NP–protein interaction. NP fate and

destination are greatly affected by the thickness, density, and affinity of the protein corona, which in turn depends on NP characteristics such as size, surface charge, physiological environment (blood, interstitial fluid, etc.) [58]. It is now clearly understood that besides size and shape of NPs, the protein corona is the other primary defining element of NP transport kinetics in biological media [59, 60]. For instance, adsorption of opsonins such as IgG, complement factors, and fibrinogen leads to removal of NPs from the systemic circulation via cells of the RES [61]. NPs adsorbed by opsonin tend to sequester in the RES organs very rapidly and concentrate in the liver and spleen [62]. In contrast, the presence of dysopsonin seems to prolong blood circulation times of NPs. For instance, albumin-bound forms of paclitaxel (Abraxane®) have been shown to target tumors because of the drug's prolonged circulation time as it avoids clearance by the RES [63].

Lundqvist et al. [64] studied the effects of NP size on the formation of the long-lived ("hard") protein corona from human plasma. To perform systematic studies of the effects of surface properties and size on the detailed protein corona, six polystyrene NPs that differed in surface chemistry (plain, carboxyl-modified, or amine-modified) and size (50 nm vs. 100 nm) were used. The study demonstrated that NP size alters the nature of the biologically active proteins in the corona. At nanoscale, the surface curvature influences protein adsorption, so that protein-binding affinities for the NP surface will differ with NP size [65].

Fibronectin has been shown to form a complex with polystyrene NPs, thereby allowing these NPs to be primarily taken up by Kupffer cells [60]. Another study showed that an increase in the amount of C3 and IgG on the surface of lecithin-coated polystyrene NPs was correlated with an increase in hepatic uptake by Kupffer cells [66]. In addition, studies have shown that the attachment of apolipoproteins (Apo), such as ApoE, ApoA-I, and ApoB-100, to poly(butylcyanoacrylate) NPs facilitates drug transport into the brain by enabling NP interaction with brain endothelial cells. In terms of specific apolipoproteins, ApoE binding was shown to be important for the transport of drugs across the blood–brain barrier [67, 68].

In general, studies have shown that the type, thickness, quantity, and conformation of the adsorbed proteins influence the protein structure on the NP surface. Therefore, it is critical to understand the relationship between NP surface characteristics and the protein corona to guide and control in vivo NP behavior [69]. Currently, several in situ or ex situ techniques are being used to analyze each of the above parameters of the protein corona. Basically, in situ techniques measure the protein corona while NPs are dispersed in a physiological environment. For instance, the thickness of the protein corona can be measured by using either dynamic light scattering or colorimetric protein assays, which measure the density of the adsorbed protein layer [70]. Most other methods used require the isolation of proteins from the NP surface.

The conformation of adsorbed proteins is measured using circular dichroism or tryptophan fluorescence quenching [71]. The affinity of NP–protein interactions can be assessed using isothermal titration calorimetry or surface plasmon resonance. Isothermal titration calorimetry provides additional information on the thermody-

namics of protein adsorption, and surface plasmon resonance provides information on adsorption kinetics [72]. Typically, either differential centrifugation or size exclusion chromatography is used for isolation of bound proteins [73]. These techniques exploit differences in the size and/or density of nanomaterials relative to the unbound protein.

Besides experimental techniques, NP–protein interactions are also studied by computer simulation studies. These simulation studies provide information about protein orientation and conformation with exceptionally high spatial and temporal resolution. Several studies have used in silico simulation to study protein adsorption to nanomaterials as a function of surface ligand structure, surface curvature, and other factors [74, 75].

However, current methods used for the biophysical characterization of NP–protein interactions also have several drawbacks. For instance, ex situ measurements require isolation of proteins; therefore they do not accurately reflect the protein corona in vivo. Very few techniques are capable of providing information on the structure of the protein corona. Current computational power is not sufficient to handle the complexity of competitive protein adsorption in a physiological environment [76]. To overcome these barriers, current research is focused on improving the techniques and strategies by which to study the protein corona with structural details and molecular resolution. There is an emerging field to characterize proteins bound to NPs as a function of their characteristics, study biodistribution, and identify which particular protein or group of proteins are responsible for targeting of NPs to a particular tissue.

20.3.2 Biophysical Characterization of NP–Cell Membrane Lipid Interactions for Targeting Cells

To develop NPs that can effectively deliver drugs to intracellular locations, one needs to understand the interactions occurring between NP and cell membranes. Recently, there is growing evidence that the cell membrane's biophysical properties influence cellular functions in regard to drug transport by NPs [7]. For instance, the cell membrane's biophysical properties have been shown to affect endocytic functions, which play a critical role in NP uptake [77, 78]. The complexity of the processes involved in NP uptake by cells and the intricacy of cell membranes make the study of these biophysical interactions more difficult to perform in live cells and in real time. Therefore, simplified artificial membrane systems, which mimic the natural bilayer lipid membrane, have been developed to study these interactions [79].

In our initial studies, we focused on understanding the interactions of NPs and cell model membranes and how these interactions correlate to cellular uptake of NPs [80]. In a subsequent series of experiments, we have studied the specificity of interactions of NPs with the membrane lipids of cancerous vs. normal cells and

whether that specificity leads to better targeting of NPs to tumor in vivo. We demonstrated a remarkable difference in biophysical interactions of the *trans*-activating transcriptor (TAT) peptide-modified and scrambled TAT peptide-modified NPs with model membrane, despite the fact that both types of NPs were of similar size and surface charge [81]. Using a Langmuir film balance, we found that TAT peptide-modified NPs showed an increase in surface pressure of the membrane, whereas the scrambled TAT peptide-modified NPs did not. Also, TAT peptide-modified NPs showed increased cellular uptake over unmodified NPs, whereas scrambled TAT peptide-modified NPs did not show any significant differences vs. unmodified NPs. In a similar set of experiments, we demonstrated that the molecular structure of cationic surfactants influences the cellular uptake of surfactant-modified NPs. Dichain didodecyldimethylammoniumbromide (DMAB)-modified NPs showed greater biophysical interaction with model membranes and greater cellular uptake than single-chain decyltrimethylammoniumbromide (DTAB)-modified NPs, although both types of NPs were of similar size and surface charge [82]. Further analysis of model membranes by atomic force microscopy following interaction with modified NPs demonstrated that DMAB-NPs penetrated the model membrane, whereas single-chain cationic cetyltrimethylammoniumbromide (CTAB)-modified NPs anchored to the membrane, suggesting their ionic interactions with anionic headgroups of lipids (Fig. 20.3). Both studies suggest that the molecular structure of the peptide and surfactant rather than the surface charge of NPs influences biophysical interactions with the lipid membrane.

Using biophysical characterization methods, we have shown that the membrane lipids of doxorubicin-resistant breast cancer cells (MCF-7/Adr) form a rigid membrane, which could be responsible for impaired endocytic function in resistant cells. On the other hand, the membrane lipids of doxorubicin-sensitive cells (MCF-7) formed a fluid membrane, which showed endocytic uptake of NPs and a dose-dependent antiproliferative effect with doxorubicin-loaded NPs. Resistant cells did not demonstrate the same dose-dependent antiproliferative effect due to impaired endocytic function in these cells, which affected intracellular drug delivery [77, 78]. The results of this study clearly suggest the significance of biophysical interactions of NPs with the cell membrane in the NP uptake process.

In a separate study, we demonstrated that DMAB-modified NPs showed greater biophysical interaction with the membrane lipids of prostate cancer cells than with those of normal endothelial cells. This specificity of interaction of DMAB-modified NPS with cancer cell lipids correlated to their increased efficacy in inhibiting tumor growth using a p53 gene-loaded modified NPs vs. unmodified p53 gene-loaded NPs [83]. To predict and understand the dynamic interactions of NP surfaces with biological environments, computational simulation techniques such as all atom Monte Carlo, molecular dynamics methods, and coarse-grained methods have recently been used [84, 85]. Based on our results, it seems feasible to explore biophysical interactions to design NPs with the ability to target particular cells/tissue.

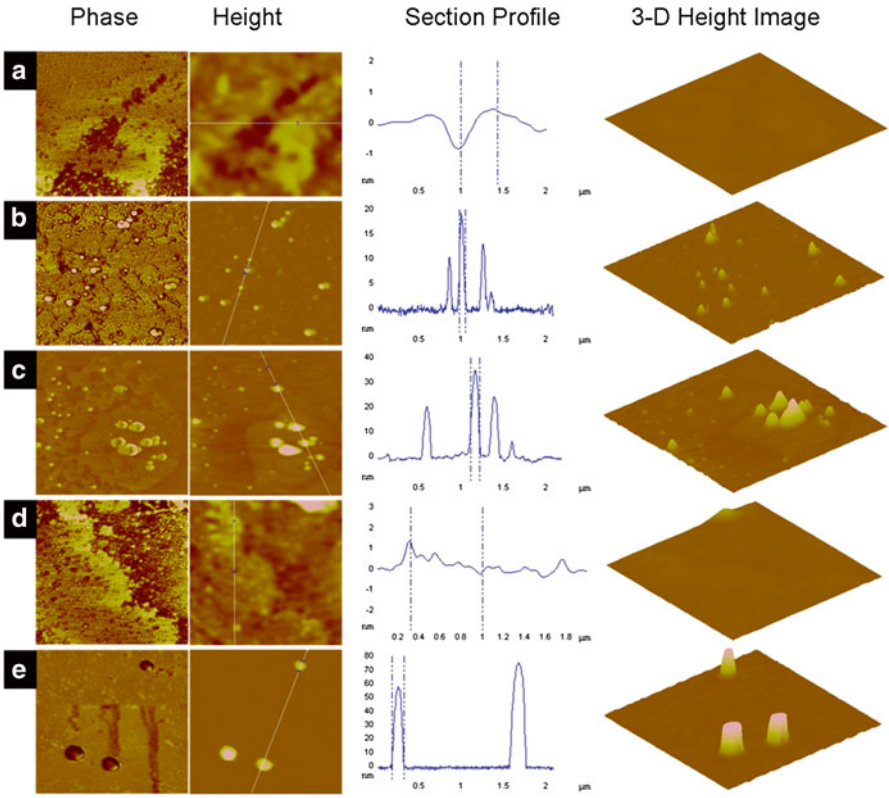

Fig. 20.3 *Surfactant structure on NP surface influences NP biophysical interactions with membrane lipids*. Changes in Langmuir-Schaefer film morphology of endothelial model membrane following interactions with different surfactant-modified polystyrene-NPs (PS-NPs). Langmuir–Schaefer films were transferred onto a silicon substrate following interaction with PS-NPs for 20 min, and imaging was carried out using tapping-mode atomic force microscopy in air. (**a**) Endothelial model membrane transferred at surface pressure 30 mN/m. (**b**, **c**, **d**, and **e**) Images of endothelial model membrane following interaction with DMAB-, CTAB-, DTAB-, and polyvinyl alcohol (PVA)-modified NPs, respectively. Phase angle scale for all images was 50°. Height scales for images: **a**, **d** = 3 nm; **b**, **c**, **f** = 50 nm; **e** = 200 nm. Section analysis was carried out on atomic force microscopic height images across the white line. Height scale for 3-D height images = 50 nm. Scan size = 2 μm. Reproduced from [82]

20.4 Impact of Physical and Biophysical Targeting Approaches on Clinical Translation of NPs

Targeting approaches based on physical and biophysical characteristics of NPs will be advantageous in clinical translation and the subsequent path to commercialization. For instance, conventional preparation of targeted NPs involves NP core formation, followed by bioconjugation with a targeting ligand to the NP surface [86]. Multiple chemical processing steps are involved in the formulation of

ligand-conjugated NPs and their purification. These processing steps influence the reproducibility of NP surface properties, resulting in batch-to-batch variability that may substantially increase the level of difficulty in predicting behavior of NPs in a biological system [87]. By developing targeting approaches using physical characteristics, one can eliminate these multiple steps in processing, thereby improving the chances of reproducibility. Indeed, using PRINT® (Particle Replication In Non-Wetting Templates, Liquidia, Research Triangle Park, NC) and other similar technologies, one can produce NPs that vary narrowly from each other in their biophysicochemical properties [39]. In addition, a physical targeting approach reduces the number of components required compared with the multicomponent nature of ligand-targeted NPs.

20.5 Conclusions

It is clearly evident that the physical characteristics of NPs play a significant role in their interactions with proteins, cells, and tissues and also influence biodistribution. Although it is a complex correlation, we have achieved some generalized understanding of NP characteristics and their effects on in vivo disposition. The complexity is partly due to variations in the NPs being investigated, which affect their interactions with the biological environment. Another cofounding variation comes from differences in the biology of target tissues or pathological variations in diseased tissues. A better understanding of the role of physical and biophysical characteristics and their significance in biodistribution in vivo could potentially offer a new approach to designing targeted NPs, ultimately improving patient care.

Acknowledgement The work described here from the authors' laboratory is funded by grant 1R01CA149359 (to V.L.) from the National Cancer Institute (NCI) of the National Institutes of Health.

References

1. Kamaly N, Xiao Z, Valencia PM, Radovic-Moreno AF, Farokhzad OC (2012) Targeted polymeric therapeutic nanoparticles: design, development and clinical translation. Chem Soc Rev 41(7):2971–3010
2. Farokhzad OC, Langer R (2009) Impact of nanotechnology on drug delivery. ACS Nano 3(1):16–20
3. Devadasu VR, Bhardwaj V, Kumar MNVR (2013) Can controversial nanotechnology promise drug delivery? Chem Rev 113(3):1686–1735
4. Sparreboom A, Scripture CD, Trieu V, Williams PJ, De T, Yang A, Beals B, Figg WD, Hawkins M, Desai N (2005) Comparative preclinical and clinical pharmacokinetics of a cremophor-free, nanoparticle albumin-bound paclitaxel (ABI-007) and paclitaxel formulated in cremophor (Taxol). Clin Cancer Res 11(11):4136–4143
5. Gradishar WJ, Tjulandin S, Davidson N, Shaw H, Desai N, Bhar P, Hawkins M, O'Shaughnessy J (2005) Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. J Clin Oncol 23(31):7794–7803

6. Campos SM, Penson RT, Mays AR, Berkowitz RS, Fuller AF, Goodman A, Matulonis UA, Muzikansky A, Seiden MV (2001) The clinical utility of liposomal doxorubicin in recurrent ovarian cancer. Gynecol Oncol 81(2):206–212
7. Peetla C, Vijayaraghavalu S, Labhasetwar V (2013) Biophysics of cell membrane lipids in cancer drug resistance: implications for drug transport and drug delivery with nanoparticles. Adv Drug Deliv Rev 65(13–14):1686–1698
8. Danhier F, Feron O, Préat V (2010) To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. J Control Release 148(2): 135–146
9. Acharya S, Sahoo SK (2011) PLGA nanoparticles containing various anticancer agents and tumour delivery by epr effect. Adv Drug Deliv Rev 63(3):170–183
10. Shi J, Xiao Z, Kamaly N, Farokhzad OC (2011) Self-assembled targeted nanoparticles: evolution of technologies and bench to bedside translation. Acc Chem Res 44(10):1123–1134
11. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ (1999) Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284(5422):1994–1998
12. Lubberts E, van den Berg WB (2003) Cytokines in the pathogenesis of rheumatoid arthritis and collagen-induced arthritis. Adv Exp Med Biol 520:194–202
13. Ruoslahti E, Bhatia SN, Sailor MJ (2010) Targeting of drugs and nanoparticles to tumors. J Cell Biol 188(6):759–768
14. Mahon E, Salvati A, Baldelli Bombelli F, Lynch I, Dawson KA (2012) Designing the nanoparticle-biomolecule interface for "targeting and therapeutic delivery". J Control Release 161(2):164–174
15. Florence AT (2012) "Targeting" nanoparticles: the constraints of physical laws and physical barriers. J Control Release 164(2):115–124
16. Morachis JM, Mahmoud EA, Almutairi A (2012) Physical and chemical strategies for therapeutic delivery by using polymeric nanoparticles. Pharmacol Rev 64(3):505–519
17. van der Meel R, Vehmeijer LJ, Kok RJ, Storm G, van Gaal EV (2013) Ligand-targeted particulate nanomedicines undergoing clinical evaluation: current status. Adv Drug Deliv Rev 65(10):1284–1298
18. Cheng ZL, Al Zaki A, Hui JZ, Muzykantov VR, Tsourkas A (2012) Multifunctional nanoparticles: cost versus benefit of adding targeting and imaging capabilities. Science 338(6109): 903–910
19. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, Bawendi MG, Frangioni JV (2007) Renal clearance of quantum dots. Nat Biotechnol 25(10):1165–1170
20. Toy R, Hayden E, Camann A, Berman Z, Vicente P, Tran E, Meyers J, Pansky J, Peiris PM, Wu HP, Exner A, Wilson D, Ghaghada KB, Karathanasis E (2013) Multimodal in vivo imaging exposes the voyage of nanoparticles in tumor microcirculation. ACS Nano 7(4):3118–3129
21. Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA (2006) In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 112(1):26–34
22. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF (2008) Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol 38(5): 1404–1413
23. Jiang W, Kim BY, Rutka JT, Chan WC (2008) Nanoparticle-mediated cellular response is size-dependent. Nat Nanotechnol 3(3):145–150
24. Schädlich A, Caysa H, Mueller T, Tenambergen F, Rose C, Göpferich A, Kuntsche J, Mäder K (2011) Tumor accumulation of NIR fluorescent PEG PLA nanoparticles: impact of particle size and human xenograft tumor model. ACS Nano 5(11):8710–8720
25. Adjei IM, Peetla C, Labhasetwar V (2014) Heterogeneity in nanoparticles influences biodistribution and targeting. Nanomedicine (Lond) 9(2):267–278
26. Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422(6927):37–44
27. Zhao F, Zhao Y, Liu Y, Chang XL, Chen CY, Zhao YL (2011) Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. Small 7(10):1322–1337

28. Wang J, Byrne JD, Napier ME, DeSimone JM (2011) More effective nanomedicines through particle design. Small 7(14):1919–1931
29. Torchilin VP (2007) Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J 9(2):E128–E147
30. Champion JA, Katare YK, Mitragotri S (2007) Making polymeric micro- and nanoparticles of complex shapes. Proc Natl Acad Sci U S A 104(29):11901–11904
31. Gratton SE, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, DeSimone JM (2008) The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci U S A 105(33):11613–11618
32. Decuzzi P, Godin B, Tanaka T, Lee SY, Chiappini C, Liu X, Ferrari M (2010) Size and shape effects in the biodistribution of intravascularly injected particles. J Control Release 141(3):320–327
33. Kolhar P, Anselmo AC, Gupta V, Pant K, Prabhakarpandian B, Ruoslahti E, Mitragotri S (2013) Using shape effects to target antibody-coated nanoparticles to lung and brain endothelium. Proc Natl Acad Sci U S A 110(26):10753–10758
34. Geng Y, Dalhaimer P, Cai S, Tsai R, Tewari M, Minko T, Discher DE (2007) Shape effects of filaments versus spherical particles in flow and drug delivery. Nat Nanotechnol 2(4):249–255
35. Barua S, Yoo JW, Kolhar P, Wakankar A, Gokarn YR, Mitragotri S (2013) Particle shape enhances specificity of antibody-displaying nanoparticles. Proc Natl Acad Sci U S A 110(9):3270–3275
36. Chauhan VP, Popović Z, Chen O, Cui J, Fukumura D, Bawendi MG, Jain RK (2011) Fluorescent nanorods and nanospheres for real-time in vivo probing of nanoparticle shape-dependent tumor penetration. Angew Chem Int Ed Engl 50(48):11417–11420
37. Arnida J-AMM, Ray A, Peterson CM, Ghandehari H (2011) Geometry and surface characteristics of gold nanoparticles influence their biodistribution and uptake by macrophages. Eur J Pharm Biopharm 77(3):417–423
38. Decuzzi P, Ferrari M (2006) The adhesive strength of non-spherical particles mediated by specific interactions. Biomaterials 27(30):5307–5314
39. Perry JL, Herlihy KP, Napier ME, Desimone JM, DeSimone JN (2011) PRINT: a novel platform toward shape and size specific nanoparticle theranostics. Acc Chem Res 44(10):990–998
40. Peiris PM, Toy R, Doolittle E, Pansky J, Abramowski A, Tam M, Vicente P, Tran E, Hayden E, Camann A, Mayer A, Erokwu BO, Berman Z, Wilson D, Baskaran H, Flask CA, Keri RA, Karathanasis E (2012) Imaging metastasis using an integrin-targeting chain-shaped nanoparticle. ACS Nano 6(10):8783–8795
41. Loverde SM, Klein ML, Discher DE (2012) Nanoparticle shape improves delivery: rational coarse grain molecular dynamics (rCG-MD) of taxol in worm-like PEG-PCL micelles. Adv Mater 24(28):3823–3830
42. Dausend J, Musyanovych A, Dass M, Walther P, Schrezenmeier H, Landfester K, Mailänder V (2008) Uptake mechanism of oppositely charged fluorescent nanoparticles in HeLa cells. Macromol Biosci 8(12):1135–1143
43. Verma A, Stellacci F (2010) Effect of surface properties on nanoparticle-cell interactions. Small 6(1):12–21
44. Salvador-Morales C, Zhang L, Langer R, Farokhzad OC (2009) Immunocompatibility properties of lipid-polymer hybrid nanoparticles with heterogeneous surface functional groups. Biomaterials 30(12):2231–2240
45. He CB, Hu YP, Yin LC, Tang C, Yin CH (2010) Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials 31(13): 3657–3666
46. Dellian M, Yuan F, Trubetskoy VS, Torchilin VP, Jain RK (2000) Vascular permeability in a human tumour xenograft: molecular charge dependence. Br J Cancer 82(9):1513–1518
47. Sankaranarayanan J, Mahmoud EA, Kim G, Morachis JM, Almutairi A (2010) Multiresponse strategies to modulate burst degradation and release from nanoparticles. ACS Nano 4(10):5930–5936

48. McNeil SE (2005) Nanotechnology for the biologist. J Leukoc Biol 78(3):585–594
49. Vonarbourg A, Passirani C, Saulnier P, Benoit JP (2006) Parameters influencing the stealthiness of colloidal drug delivery systems. Biomaterials 27(24):4356–4373
50. Vonarbourg A, Passirani C, Saulnier P, Simard P, Leroux JC, Benoit JP (2006) Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake. J Biomed Mater Res A 78(3):620–628
51. Aggarwal P, Hall JB, McLeland CB, Dobrovolskaia MA, McNeil SE (2009) Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. Adv Drug Deliv Rev 61(6):428–437
52. Hoek EMV, Agarwal GK (2006) Extended DLVO interactions between spherical particles and rough surfaces. J Colloid Interface Sci 298(1):50–58
53. Nel AE, Madler L, Velegol D, Xia T, Hoek EMV, Somasundaran P, Klaessig F, Castranova V, Thompson M (2009) Understanding biophysicochemical interactions at the nano-bio interface. Nat Mat 8(7):543–557
54. Verhoef JJF, Anchordoquy TJ (2013) Questioning the use of PEGlyation drug delivery. Drug Deliv Transl Res 3(6):499–503
55. Neal JC, Stolnik S, Schacht E, Kenawy ER, Garnett MC, Davis SS, Illum L (1998) In vitro displacement by rat serum of adsorbed radiolabeled poloxamer and poloxamine copolymers from model and biodegradable nanospheres. J Pharm Sci 87(10):1242–1248
56. Zahr AS, Davis CA, Pishko MV (2006) Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol). Langmuir 22(19):8178–8185
57. Wu YL, Putcha N, Ng KW, Leong DT, Lim CT, Loo SCJ, Chen XD (2013) Biophysical responses upon the interaction of nanomaterials with cellular interfaces. Acc Chem Res 46(3):782–791
58. Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA (2008) Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci U S A 105(38):14265–14270
59. Chithrani BD, Ghazani AA, Chan WCW (2006) Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett 6(4):662–668
60. Dutta D, Sundaram SK, Teeguarden JG, Riley BJ, Fifield LS, Jacobs JM, Addleman SR, Kaysen GA, Moudgil BM, Weber TJ (2007) Adsorbed proteins influence the biological activity and molecular targeting of nanomaterials. Toxicol Sci 100(1):303–315
61. Göppert TM, Müller RH (2005) Adsorption kinetics of plasma proteins on solid lipid nanoparticles for drug targeting. Int J Pharm 302(1–2):172–186
62. Camner P, Lundborg M, Låstbom L, Gerde P, Gross N, Jarstrand C (2002) Experimental and calculated parameters on particle phagocytosis by alveolar macrophages. J Appl Physiol 92(6):2608–2616
63. Ogawara K, Furumoto K, Nagayama S, Minato K, Higaki K, Kai T, Kimura T (2004) Precoating with serum albumin reduces receptor-mediated hepatic disposition of polystyrene nanosphere: implications for rational design of nanoparticles. J Control Release 100(3):451–455
64. Lundqvist M, Stigler J, Cedervall T, Berggård T, Flanagan MB, Lynch I, Elia G, Dawson K (2011) The evolution of the protein corona around nanoparticles: a test study. ACS Nano 5(9):7503–7509
65. Lundqvist M, Sethson I, Jonsson BH (2004) Protein adsorption onto silica nanoparticles: conformational changes depend on the particles' curvature and the protein stability. Langmuir 20(24):10639–10647
66. Nagayama S, Ogawara K, Fukuoka Y, Higaki K, Kimura T (2007) Time-dependent changes in opsonin amount associated on nanoparticles alter their hepatic uptake characteristics. Int J Pharm 342(1–2):215–221
67. Kreuter J, Hekmatara T, Dreis S, Vogel T, Gelperina S, Langer K (2007) Covalent attachment of apolipoprotein A-I and apolipoprotein B-100 to albumin nanoparticles enables drug transport into the brain. J Control Release 118(1):54–58

68. Michaelis K, Hoffmann MM, Dreis S, Herbert E, Alyautdin RN, Michaelis M, Kreuter J, Langer K (2006) Covalent linkage of apolipoprotein e to albumin nanoparticles strongly enhances drug transport into the brain. J Pharmacol Exp Ther 317(3):1246–1253
69. Walkey CD, Chan WC (2012) Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. Chem Soc Rev 41(7):2780–2799
70. Walczyk D, Bombelli FB, Monopoli MP, Lynch I, Dawson KA (2010) What the cell "sees" in bionanoscience. J Am Chem Soc 132(16):5761–5768
71. Deng ZJ, Liang M, Monteiro M, Toth I, Minchin RF (2011) Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation. Nat Nanotechnol 6(1): 39–44
72. Lindman S, Lynch I, Thulin E, Nilsson H, Dawson KA, Linse S (2007) Systematic investigation of the thermodynamics of HSA adsorption to N-iso-propylacrylamide/N-tert-butylacrylamide copolymer nanoparticles. Effects of particle size and hydrophobicity. Nano Lett 7(4): 914–920
73. Cedervall T, Lynch I, Lindman S, Berggård T, Thulin E, Nilsson H, Dawson KA, Linse S (2007) Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc Natl Acad Sci U S A 104(7): 2050–2055
74. Ge C, Du J, Zhao L, Wang L, Liu Y, Li D, Yang Y, Zhou R, Zhao Y, Chai Z, Chen C (2011) Binding of blood proteins to carbon nanotubes reduces cytotoxicity. Proc Natl Acad Sci U S A 108(41):16968–16973
75. Zuo G, Huang Q, Wei G, Zhou R, Fang H (2010) Plugging into proteins: poisoning protein function by a hydrophobic nanoparticle. ACS Nano 4(12):7508–7514
76. Iori F, Di Felice R, Molinari E, Corni S (2009) GoIP: an atomistic force-field to describe the interaction of proteins with Au(111) surfaces in water. J Comput Chem 30(9):1465–1476
77. Peetla C, Bhave R, Vijayaraghavalu S, Stine A, Kooijman E, Labhasetwar V (2010) Drug resistance in breast cancer cells: biophysical characterization of and doxorubicin interactions with membrane lipids. Mol Pharm 7(6):2334–2348
78. Vijayaraghavalu S, Peetla C, Lu S, Labhasetwar V (2012) Epigenetic modulation of the biophysical properties of drug-resistant cell lipids to restore drug transport and endocytic functions. Mol Pharm 9(9):2730–2742
79. Peetla C, Stine A, Labhasetwar V (2009) Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery. Mol Pharm 6(5):1264–1276
80. Peetla C, Labhasetwar V (2008) Biophysical characterization of nanoparticle-endothelial model cell membrane interactions. Mol Pharm 5(3):418–429
81. Peetla C, Rao KS, Labhasetwar V (2009) Relevance of biophysical interactions of nanoparticles with a model membrane in predicting cellular uptake: study with TAT peptide-conjugated nanoparticles. Mol Pharm 6(5):1311–1320
82. Peetla C, Labhasetwar V (2009) Effect of molecular structure of cationic surfactants on biophysical interactions of surfactant-modified nanoparticles with a model membrane and cellular uptake. Langmuir 25(4):2369–2377
83. Sharma B, Peetla C, Adjei IM, Labhasetwar V (2013) Selective biophysical interactions of surface modified nanoparticles with cancer cell lipids improve tumor targeting and gene therapy. Cancer Lett 334(2):228–236
84. Reynwar BJ, Illya G, Harmandaris VA, Müller MM, Kremer K, Deserno M (2007) Aggregation and vesiculation of membrane proteins by curvature-mediated interactions. Nature 447(7143):461–464
85. Roiter Y, Ornatska M, Rammohan AR, Balakrishnan J, Heine DR, Minko S (2008) Interaction of nanoparticles with lipid membrane. Nano Lett 8(3):941–944
86. Duncan R, Gaspar R (2011) Nanomedicine(s) under the microscope. Mol Pharm 8(6):2101–2141
87. Desai N (2012) Challenges in development of nanoparticle-based therapeutics. AAPS J 14(2):282–295

Chapter 21
In Vivo Imaging Techniques of the Nanocarriers Used for Targeted Drug Delivery

Sudha Rana, Amit Tyagi, Nabo Kumar Chaudhury, and Rakesh Kumar Sharma

21.1 Introduction

There has been keen interest in the development of a novel drug delivery system with aims to deliver the drug at a rate needed by the body during the period of treatment, and also to direct the active entity to the site of action. However, at present, no available drug delivery system behaves ideally achieving all the lofty goals, a sincere attempt is underway to achieve them through different novel approaches in drug delivery [1, 2]. Among them, encapsulation or entrapment of the drug in vesicular or microparticulate/nanoparticulate structures is one such system widely investigated actively over the last 3–4 decades. These systems are designed with possibility to prolong the existence of the drug in systematic circulation, and reduce the toxicity, if selective uptake can be achieved [3–5]. Many limitations identified in conventional chemotherapy like ineffective permeation across biomembranes, lack of selective uptake leading to very high volume of distribution in the body could be overcome by use of targeted drug delivery (TDD) systems [6]. Particulate systems, such as nanoparticles and dendrimers could be used for site-specific and sustained delivery of drugs. Such delivery systems will likely modify the disposition of drugs at the target site, sustain drug release, reduce toxicity to the other tissues, and increase intracellular levels of the drug. Advent of the nanotechnology paved the way to develop various nanoparticles prepared out of polymers, lipids,

S. Rana • R.K. Sharma (✉)
Division of CBRN Defence, Institute of Nuclear Medicine and Allied Sciences, Delhi, India
e-mail: rksharmadrl@yahoo.com; rks@inmas.drdo.in

A. Tyagi
Division of Nuclear Medicine, Institute of Nuclear Medicine and Allied Sciences, Delhi, India

N.K. Chaudhury
Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi, India

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_21

inorganic substances like calcium phosphate, silicates and from carbon nanotubes. Nanocarriers are playing a major role in modelling biological membranes and in the transport and targeting medicinal agent for the TDD.

Noninvasive imaging techniques are becoming popular in drug delivery/evaluation process for target organ. These methods are including gamma scintigraphy, functional magnetic resonance imaging (fMRI/MRI), X-ray computed tomography, optical imaging, positron emission tomography (PET), and fluorescence imaging. In PET, the radionuclides used are elements such as carbon (^{11}C), oxygen (^{15}O), nitrogen (^{13}N), and fluorine (^{18}F), which have made the technique of pharmacoscintigraphy broader in application but, at the same time, more expensive for routine purposes. On the other hand, MRI exploits the differences in relaxation rates of water protons in tissues, translating them into three-dimensional anatomical information. MRI is generally used to generate anatomical information; however, during last 10 years, MRI has demonstrated impressive capabilities for perfusion imaging. At the present time, the capabilities for MRI imaging of biochemical processes, such as receptor binding, have not yet been demonstrated. Figure 21.1 represents the different advanced noninvasive in vivo imaging technologies.

The field of nuclear medicine has progressed tremendously. Application of the short-lived radiation emitting radionuclides in routine medicine for therapeutic or diagnostic purposes is well established. In vivo imaging using radionuclides has been studied for the determination of physiology rather than the anatomy, which serves the purpose of being functional in nature. Gamma radiation has also been a part of this progress over the years for drug delivery assessment. The quantitative

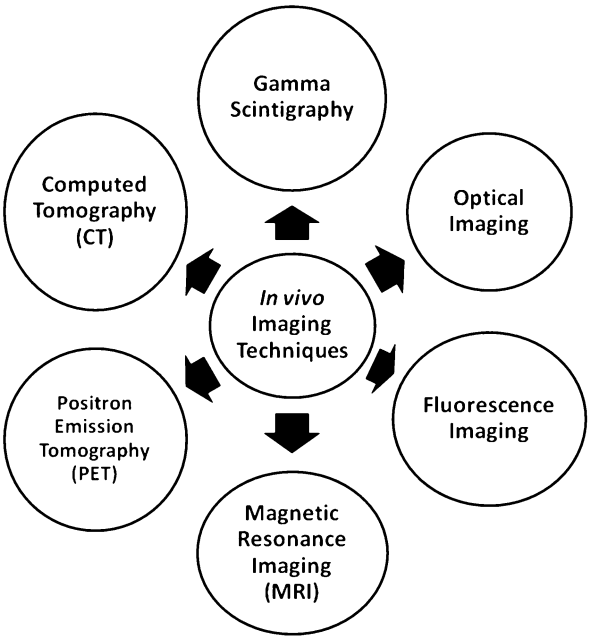

Fig. 21.1 Representing noninvasive in vivo imaging techniques for the evaluation of the nanocarrier based targeted drug delivery

nature of the imaging techniques makes them a viable tool for investigation, wherein correlations can be drawn regarding the pharmacology of the molecule, the pharmacokinetics related, and in drawing a proof of concept for a delivery system in a targeted organ. The reliability and reproducibility of the imaging techniques employed make them an acceptable technique in the advent future.

21.2 Nanocarriers

Nanotechnology-based drug delivery systems (i.e., nanoparticles and liposomes) have been investigated at large. Nanocarriers are nanomaterial being used as a transport module for targeted drug delivery TDD, useful in the drug delivery process because they can deliver drugs to specific sites, certain cells or organs but not in others. Their unique characteristics demonstrate potential use in chemotherapy drug interaction with the diseased tissues. The advantages of this system is the reduction in the frequency of the dosages taken by the patient, having a more uniform effect of the drug with reduced drug's side effects and fluctuation in circulating drug levels [7]. Nanocarriers such as polymer conjugates, polymeric nanoparticles, lipid-based carriers, dendrimers, carbon nanotubes, gold nanoparticles, gold nanoshells and nanocages are currently using for TDD [8, 9]. TDD using nanocarriers to its site of action has been attributed to a significant reduction in the drug toxicity, and reduces the dose required by the individual at the same time. As a result, the therapeutic goals can be achieved with significantly less amounts of drug on a weight basis. In cases where site targeting of drugs is performed, it is quite difficult to assess pharmacokinetics at the site of action or at the level of the targeted organ. In such cases, where the process of assessment is a challenge, scintigraphic techniques can help propel the development of novel products that otherwise would fail at some point in the development pipeline. Another factor responsible for the failure of novel products is the inability of innovators to develop an in vitro—in vivo co-relationship for the complex release mechanism of these products. This can be overcome by scintigraphic techniques, which enable us in demonstrating the release mechanisms in a more realistic way.

21.2.1 Nanocarrier Targeted Drug Delivery

The use of nanoparticulate carriers to enhance the in vivo efficiency of many drugs is well established itself over the past few decades. Surface modification of pharmaceutical nanocarriers such as liposome, micelles, nanocapsules, polymeric nanoparticles, solid lipid particles, niosomes, and others is commonly used for delivery of drugs in different targeted organs to improve its effectiveness. Nanocarrier TDD provides an opportunity to provide maximum chance of success of simultaneously perform various therapeutically or diagnostically important functions.

The most important results of such modification include an increased stability and half-life of nanocarriers in the circulation, specific biodistribution, passive/active targeting into the targeted organ, responsiveness to local physiological stimuli such as pathology-associated changes in local pH and/or temperature, and ability to serve as imaging/contrast agents for various imaging modalities (gamma scintigraphy, magnetic resonance imaging, computed tomography, fluorescence).

Currently used pharmaceutical nanocarriers such as liposomes, micelles, nanoemulsions, polymeric nanoparticles, and others demonstrate a variety of characteristics such as longevity/retention in the blood allowing for their accumulation in targeted/pathological areas or organs with compromised vasculature. TDD involves various specific targeting ligands attached to the surface of the nanocarriers that enhanced intracellular penetration with the help of surface-attached cell-penetrating molecules, contrast properties due to the carrier loading with various contrast materials allowing for direct carrier visualization in vivo and stimuli-sensitivity allowed for drug release from the carriers under certain physiological conditions. Similarly, the engineering of multifunctional pharmaceutical nanocarriers combining several useful properties in one particle can significantly enhance the efficacy of many therapeutic and diagnostic procedures. Site-specificity is a major therapeutic benefit avoids from being delivery of drugs to the wrong places. Nanocarriers show promise for use in chemotherapy because they can decrease the adverse, broader-scale toxicity on healthy. Generally most of the chemotherapy drugs are extremely toxic in nature obligate to deliver the drugs to targeted organs, e.g., tumor or brain without being released into other parts of the body. Nanocarrier based drug delivery for the hydrophobic drugs are major advantages because the human body contains mostly water is a major therapeutic benefit. Some nanocarriers contain nanotube arrays that may allow them to deliver both hydrophobic and hydrophilic drugs [10–12].

21.3 In Vivo Noninvasive Imaging

Noninvasive imaging techniques have provided valuable insights into a variety of complex systems and processes and increasingly being used in basic, preclinical and clinical research. It allows measurement of tiny changes in the body. Imaging technologies have improved their sensitivities to image different types of noninvasive cancers such as ovarian cancer, breast cancer by using the noninvasive imaging technologies such as gamma scintigraphy, MRI, PET, CT, fluorescence, and optical imaging. Since these devices are almost identical for animals and humans, they are becoming increasingly popular. Photoacoustic Tomography (PAT) is a rapidly emerging noninvasive imaging technology that integrates the merits of high optical contrast with high ultrasound resolution. Multifunctional and water-soluble super paramagnetic iron oxide (SPIO) nanocarriers are developed for targeted drug delivery using PET/MRI, routinely used as a tool of clinical radiology [13–16]. These techniques provide a handy tool for evaluating targeted drug delivery using

novel drug carrier systems. This paper considers current status and possible future directions in the emerging area of multifunctional nanocarriers those are specifically studied for specific characteristics as longevity, targetability, intracellular penetration, and contrast loading. Figure 21.2 represents the different modes of drug targeting for tumor.

21.4 In Vivo Noninvasive Imaging Techniques

Advanced noninvasive in vivo imaging technologies used for TDD are presented in and briefly discussed here as follows:

21.4.1 Gamma Scintigraphy Technique

Gamma scintigraphy imaging technique has been used in medicine for diagnostic or therapeutic purposes, an emerging and advanced technology. Over the last few years, this functional imaging technique has been utilized for the evaluation of targeted drugs/drug delivery systems that provides rapid and accurate information of drugs. The principle of this technique involves tagging of suitable γ-emitting/positron-emitting radionuclide with the active constituent or excipient of the formulation. The dosage form is then administered via the intended route and the subject is scanned under a gamma camera. Thus, real time information regarding the site, extent and rate of drug release could be obtained giving qualitative (scintigraph) and quantitative data (radioactivity counts). Gamma scintigraphy method presents the best opportunity for characterization of fluid distributions in 2D and 3D at the Darcy scale.

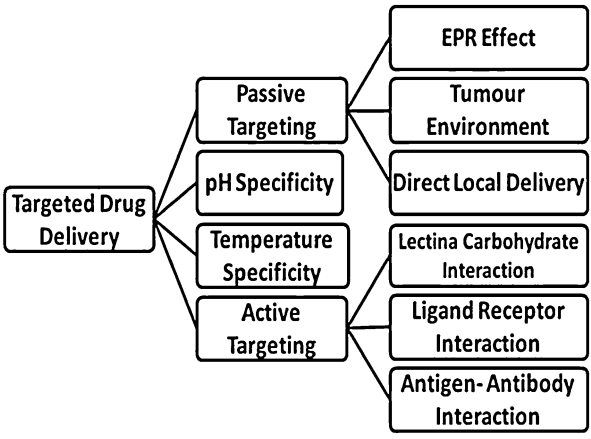

Fig. 21.2 Tumor targeted drug delivery

Technetium (^{99mTc}) is one of the most popular isotopes. It has a relatively short but optimum half-life of 6 h, during which the imaging can be completed. On the other hand, thorium (^{201}Th) is not used despite its very long half-life of 3 days. Short half-life isotopes, such as krypton (^{81m}Kr), do not allow the preparation of the test formulation and subsequent administration and imaging to be carried out. Moreover, the signal–noise ratio drops to a level where the later images are of poor quality. To overcome this problem, initial activity levels may be rose, which results in a larger absorbed radiation dose for the subject. On the other hand, isotopes with a longer half-life also show a greater absorbed radiation dose. For the purpose of pharmacoscintigraphy, another class of isotopes that can be used includes those that do not spontaneously emit gamma radiation, but are activated by a neutron flux to release gamma radiation. Both enriched and naturally abundant forms are used in neutron activation–based gamma scintigraphy. Table 21.1 lists radionuclides commonly used in traditional gamma scintigraphy.

A good marker for neutron activation-based gamma scintigraphy must possess certain properties in that (1) the cross-section for neutron capture must be large, (2) the gamma quanta emitted by the daughter nuclide must have energies suitable for gamma imaging (100–400 keV), and (3) neutron irradiation must not produce significant amounts of alpha or beta-emitting daughter nuclides. The most commonly used isotopes for neutron activation–based gamma scintigraphy are barium (^{138}Ba), samarium (^{152}Sm), and erbium (^{170}Er). These isotopes are expensive for research purposes, and hence natural abundant forms have been used with good results.

Nanotechnology-based nanocarriers such nanoparticles and liposomes are being investigated for various novel applications at large scale. In cases where site targeting of drugs have to perform is quite difficult to assess pharmacokinetics at the site of

Table 21.1 Properties of the radionuclides commonly used in traditional gamma scintigraphy

S. no.	Radionuclide	Type of decay	Principal photon energy (keV)	Physical half-life	Type(s) of radiation emitted	Mode of decay
1	Technetium (^{99m}Tc)	Electron capture	140	6 h	γ radiation	-to ^{99}Ru
2	Krypton (^{81m}Kr)	Isomer transition	191	13 s	positron, γ radiation and β particles	-to ^{83}Ru
3	Indium (^{111}In)	Electron capture	173, 247	2.8 days	β particles	-to ^{201}Pb
4	Iodine (^{123}I)	Electron capture	160	13 h	γ radiation and β particles	–to ^{131}Xe
5	Iodine (^{131}I)	Beta emission	360	8 days	γ radiation and β particles	–to ^{131}Xe
6	Thorium (^{201}Th)	Electron capture	78	73.1 h	α and β particles	-to $^{\mathbf{201}}$Pb
7	Thallium (^{201}Tl)	Gamma emission	135–167	72.912 h	γ radiation	–to ^{201}Pb

action or at the level of the targeted organ by conventional techniques. In such cases, scintigraphic technique can be used which enables us to demonstrate the release mechanisms in a more realistic way. Another approach is targeting the drug(s) to its site of action, attributing it to a significant reduction in drug toxicity, and reducing the dose required by an individual at the same time.

Gamma scintigraphy offers a great advantage over conventional pharmacokinetic methods for assessment of inhaled drug delivery systems for differences in regional lung deposition. Researchers worldwide have made use of gamma scintigraphy [17]. The most efficient method currently available for assessing the equivalence of inhaled medications in the lung region (central, intermediate, and peripheral sections) which branched into the respiratory tree, mixed, and alveolar region [18]. Ventilation scintigraphy technique involves radiolabeling of the drug, usually with ^{99m}Tc, validating the particle size of the drug. After radiolabeling the drug with suitable radiotracer, the respiratory fraction for any inhaled medication, i.e., the fraction of drug deposited in the central, intermediate, and peripheral lung, can then be calculated from serial scintigraphic scans. Visual comparison between the lung images can be done to record movement of the deposited drug with time from one compartment to another. This technique has been successfully used in development, characterization, and comparison of a number of respiratory nano-formulations [19, 20].

Drug delivery to ocular region is a challenging task because only 1–2 % of drug remains available in eye for therapeutic action and rest of the drug drains out through nasolachrymal drainage system and other ocular physiological barriers. To overcome these problems of conventional dosage form, novel ocular drug delivery systems are being explored all over the world. Pharmacoscintigraphy can be a powerful tool to evaluate ocular drug delivery systems too. Precorneal retention and lacrimal clearance could be studied successfully employing this technique. Pharmacoscintigraphy can be used to study the exact site of release, integrity of the formulation and percentage of drug released and absorbed into the systemic circulation. Figure 21.3 is depicting the Atropine sulfate-^{99m}Tc DTPA formulation release studies and real time absorption into the systemic circulation using Pharmacoscintigraphy technique. Figure 21.4 is the presentation of gastric emptying studies after oral administration of 99mTc-DTPA suspension employing scintigraphic technique [21] (data unpublished).

Site-specific targeted drug delivery negotiates an exclusive delivery to specific pre-identified compartment with maximum intrinsic activity of the drug and concomitantly reduced access of the drug to other irrelevant non-target cells/sites. A number of strategies are followed to target various body tissues and organs such as liposomes, colloidal systems, and dendrimers for TDD research. Incorporation of drugs into delivery carriers prevents degradation, targets drugs to the site of action, and reduces toxicity or side effects by modifying their in vivo distribution. In the process of targeting, systemic toxicity is avoided and the amount of drug reaching the site of action is also increased. As a consequence, the required dose may be reduced. Active investigation in this field has been observed in recent years. 117 Radiolabeling of drugs and their carriers to study their in vivo distribution has been widely attempted. In addition to being noninvasive, this evaluation method also has the

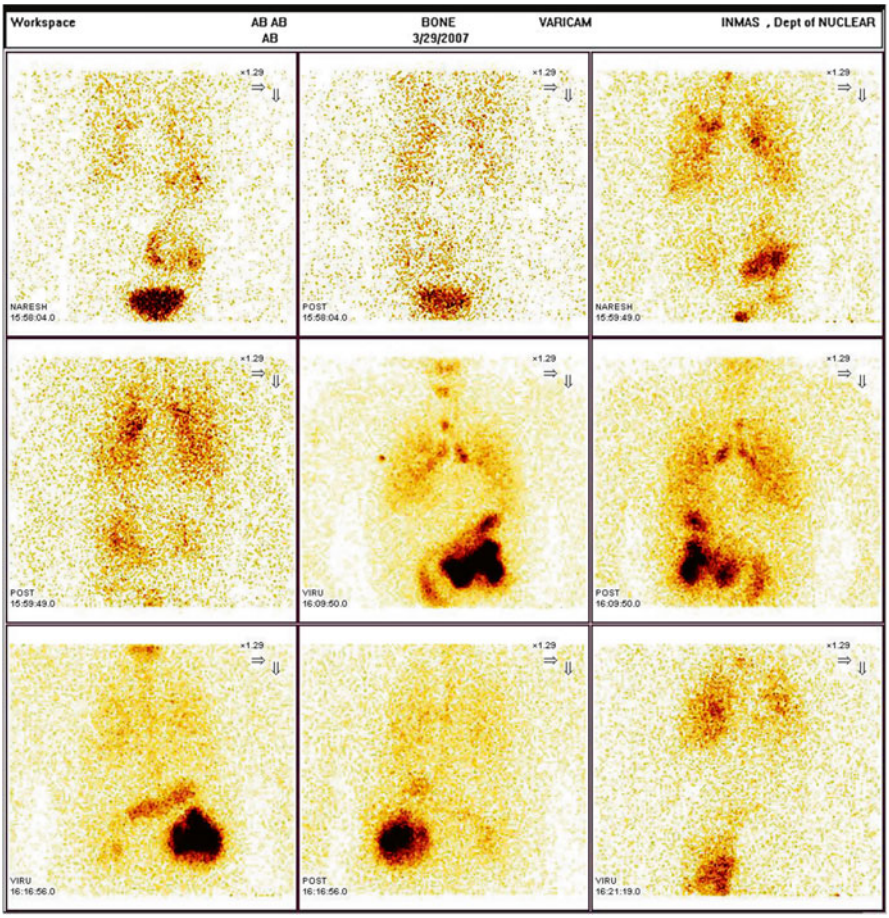

Fig. 21.3 Atropine sulfate-^{99m}Tc DTPA formulation release studies and real time absorption into the systemic circulation using pharmacoscintigraphy technique

advantage of monitoring the movement of the formulation to the targeting site, where it remains localized. The model drug, an antiretroviral called stavudine, is rapidly excreted by the renal route and the scintigrams observed were in accordance with this fact. When a liposome-based formulation was used, the formulation was evenly accumulated in the liver and spleen, which reduced the renal excretion of the drug.

21.4.2 X-Ray Computed Tomography Imaging Technique

X-ray computed tomography (CT) is a well-established noninvasive imaging technique employed for a variety of research as well as clinical applications. Specifically, CT imaging as a diagnostic tool allows for 3D visual reconstruction

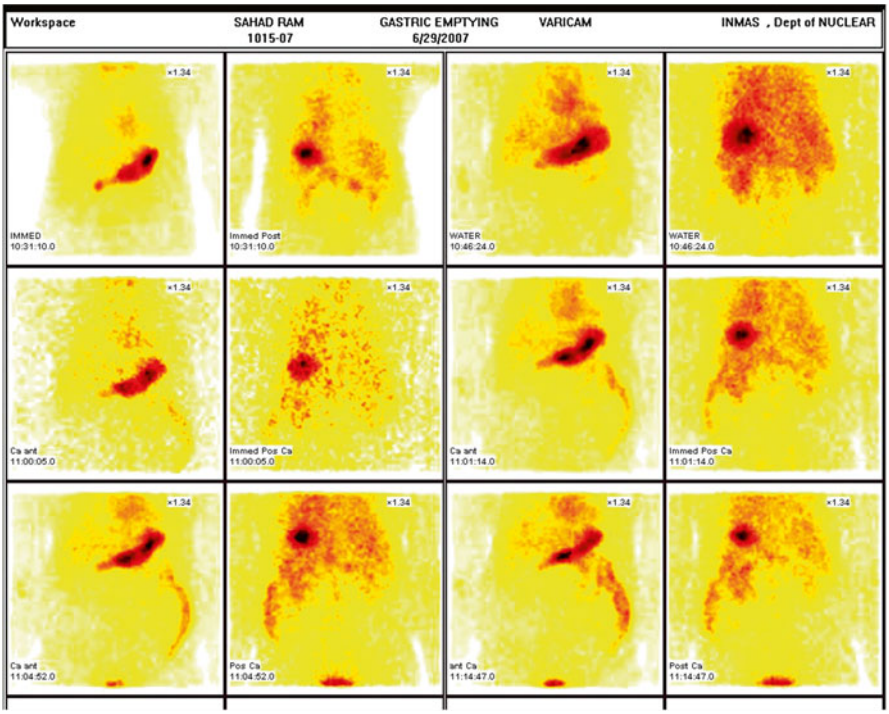

Fig. 21.4 Scintigraphic evaluation of gastric emptying after oral administration of 99mTc-DTPA suspension

and segmentation of the tissues of interest. High resolution CT systems can be used to perform non-destructive 3D imaging of a variety of tissue types and organ systems, e.g., gastrointestinal tract, cardiovascular system, renal tract, liver, lungs, bone, cartilage, tumorous tissue. It is one of the most prevalent diagnostic tools in terms of frequency-of-use and hospital availability. The CT contrast agent should localize or target the tissue of interest, improve the visualization of the target tissue, its longer tissue retention-time possess favorable bio-distribution and pharmacokinetic profiles. Contrast agent should be readily soluble or form stable suspensions at aqueous physiological conditions at appropriate pH and osmolality with low viscosity with least toxicity of metabolites. In order to achieve higher levels of X-ray attenuation than observed for biological tissue, elements of higher atomic number (Z) are incorporated into the contrast agent molecule. Iodine ($Z=53$) has historically been the atom of choice for CT imaging applications. Sodium and lithium iodide were among the first water-soluble imaging agents [14].

Nanoparticles preferably accumulate in solid tumors by means of passive convective transport through leaky endothelium (extravasation) with the pore size of approximately 100–800 nm enhance the permeation and retention effect. PEGylated liposomes (100 nm), carrying iodinated contrast agents such as iopamidol, or iodixanol can be used as blood-pool imaging agents for visualization of breast tumor lesions.

Utility of these liposomal contrast agents for imaging of tumor angiogenesis was successfully demonstrated in rodents. The X-ray imaging obtained can be used to predict the proper therapeutic gap filling and the effective of treatment by nano filled composite resin as presented in Fig. 21.5 [22] (data unpublished).

Gold nanoparticles (AuNPs) are an ideal radiopaque nanoparticulate contrast media because gold has both a high density and a high atomic number and thus AuNPs possess favorable X-ray attenuating properties. Gold provides about 2.7 times greater contrast per unit weight than iodine. AuNPs have been investigated since many years as a nanocarrier for the targeted drug delivery and also for other clinical applications. AuNPs are using widely due to the relative ease of synthesis and good control over their size, ease of surface modification with various biologically or diagnostically important molecules along with and good bio-tolerability and non-toxicity [23]. Current clinical CT scanners are capable of acquiring high resolution 3D isotropic images of the body within few minutes, less time consuming, less expensive and more readily available than other medical imaging technologies like MRI and PET.

Although CT contrast agents offer safety and imaging efficacy, they do suffer from several drawbacks, which prevent them from being used for all applications:

- They exhibit nonspecific biodistribution.
- Due to their relatively small size they tend to undergo rapid renal clearance from the body.
- The often high osmolality and/or high viscosity of the contrast media formulations can lead to renal toxicity and adverse physiological effects.

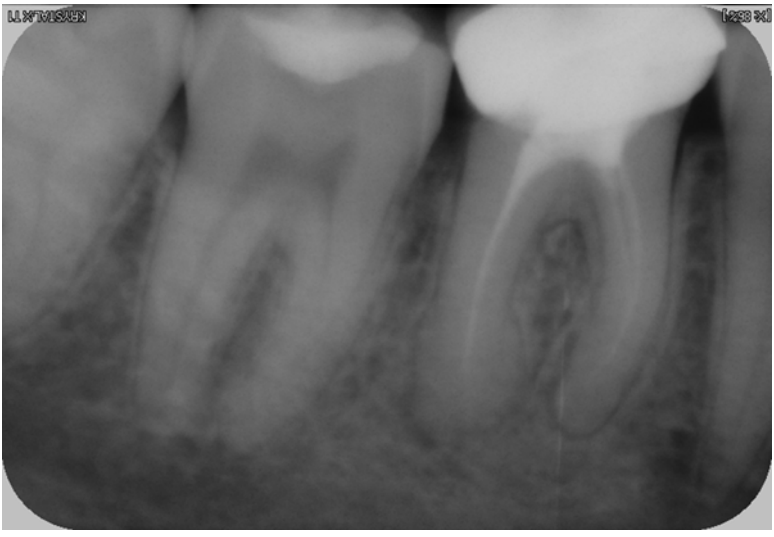

Fig. 21.5 Radiograph (RVG) of tooth restored with nano filled composite resin

– High "per dose" concentrations are required.
– High rates of extravasation and equilibration between intravascular and extravascular compartments at the capillary level often make it difficult to obtain meaningful and clear CT images.

21.4.3 Magnetic Resonance Imaging (MRI) Technique

MRI is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation energy allowing the observation of magnetic properties of the atomic nucleus. Typically MRI is not capable of sensing biochemical activities. However, recently emerged activatable MRI contrast agents (CAs), whose relaxivity is variable in response to a specific parameter change in the surrounding physiological microenvironment, potentially allow for MRI to indicate biological processes. MRI provides anatomical images by measuring proton (^{1}H) relaxation processes of water and soft tissues in biological systems. MRI is routinely used in advanced medical imaging for disease diagnosis and treatment. Most commonly used compounds for contrast enhancement is gadolinium-based which alters the relaxation times of atoms within body tissues where they are present after oral or intravenous administration. In MRI scanners body exposed to a very strong magnetic field then a radiofrequency pulse is applied causing some atoms (including those in contrast agents) to spin and then relax after the pulse stops. This relaxation emits energy which is detected by the scanner and is mathematically converted into an image. The MRI image can be weighted in different ways giving a higher or lower signal [8].

Certain contrast agents are predominantly used to shorten the T(1) relaxation time and these are mainly based on low-molecular weight chelates of the gadolinium ion (Gd^{3+}). The most widely used T(2) shortening agents are based on iron oxide (FeO) particles. Depending on their chemical composition, molecular structure and overall size, the in vivo distribution volume and pharmacokinetic properties vary widely between different contrast agents and these largely determine their use in specific diagnostic tests.

A number of MR modalities using either endogenous or exogenous agents are a more complete, less-invasive alternative to microelectrode-based pH measurements. Superparamagnetic iron oxide nanoparticles (SPIONs) are a class of MRI contrast agents having high biological tolerability and large magnetic moments, giving rise to high relaxivities, up to ~10^{2}–10^{3} $mM^{-1}s^{-1}$ per Fe ion). SPIONs are synthesized with surface modifications to improve aqueous solubility/stability, limit aggregation, and modulate biological uptake that improve the information content of the SPIONs' MR response, thereby targeting specific tissue types. MRI presents the best option for 3D characterization of fluid distribution, fluid flow, colloid transport, and reaction in artificial porous media.

Iron oxides (hydroxides or oxy-hydroxides including $Fe(OH)_3$, $Fe(OH)_2$, $Fe_5HO_8{\cdot}4H_2O$, Fe_3O_4, FeO, five polymorphs of FeOOH and four of Fe_2O_3) are one of the most important transition metal that have been studied for the drug delivery. Characteristics of these oxide compounds include mostly the trivalent state of the iron, low solubility, and brilliant colors. All the iron oxides are crystalline (except Schwertmannite and ferrihydrite) can be synthesized by all known wet chemical methods. But tailoring these particles size in nano range and morphology towards a particular application still remains a challenging task.

Diagnostic application of MRI imaging is the main use. The nano size of magnetic particle with large surface area change some of the magnetic properties and exhibit superparamagnetic characteristics quantum tunnelling of magnetization which offer a high potential for several biomedical applications. Aqueous magnetic fluids with small magnetic particles (about 5–20 nm) covered with biocompatible functionalized shells were analyzed against a number of diseases, e.g., hyperthermia, immunoassays, imaging of organs, and as magnetic nanocarriers for identification and isolation of blood cells and antibodies and for drug targeting. With proper surface coating, these magnetic nanoparticles can be dispersed into suitable solvents, forming homogeneous suspensions, known as ferrofluids, interact with an external magnetic field, and be positioned to a specific area, facilitating magnetic resonance imaging for medical diagnosis as well as AC magnetic field-assisted cancer therapy [24].

Magnetic nanoparticles have recently been used to treat bone diseases by using surface modified magnetic nanoparticles such as osteoporosis and infection where magnetite (Fe_3O_4) and maghemite (Fe_2O_3) coated with calcium phosphate (CaP). Various applications of iron oxides/hydroxides/oxyhydroxides in nano form can be specific due to the difference in behavior of particles in nano scale when compared to their bulk counterparts. The nano particles usually have much larger surface area due to their smaller size and can reduce the volume required to achieve same effect when used as a catalyst. In case of mitigation of anions/cations from aqueous solutions, iron oxides in nano form will have higher number of active sites for adsorption, thereby reducing the amount required per liter of solution. The adsorption process involves surface hydroxyl group interaction with adsorbents.

Optical based analysis can be used to visualize functional properties of various systems, e.g., changes in blood volume, oxygen consumption, and cellular swelling in relation to brain physiology and pathology. Nano iron oxides exhibit very different magnetic properties which can be used for soft ferrites and biomedical applications including TDD and magnetic resonance imaging. Down to the nanoscale, superparamagnetic iron oxide nanoparticles can only be magnetized in the presence of an external magnetic field, which makes them capable of forming stable colloids in a physio-biological medium. Their superparamagnetic property together with other intrinsic properties such as low cytotoxicity, colloidal stability, and bioactive molecule conjugation capability, makes such nanomagnets ideal in both in vitro and in vivo biomedical applications. In contrast to CT and PET imaging, MRI uses no ionizing radiation and it is therefore often deemed safer than the other two.

21.4.4 Positron Emission Tomography (PET) Imaging Technique

PET is a nuclear medicine, functional imaging technique that produces a three-dimensional color image of the functional processes that uses a radioactive substance called a tracer to look for disease in the body. It is a specialized radiology procedure used to examine various body tissues to identify certain conditions. *Positrons are using as imaging agents and probes in nanotechnology i*ntroduced in the early 1970s. It has now developed into a powerful medical diagnostic tool for routine clinical use as well as in drug development. Unequalled as a highly sensitive, specific and noninvasive imaging tool, PET unfortunately lacks the resolution of Computer Tomography (CT) and MRI. As the resolution of PET depends significantly on the energy of the positron incorporated in the radiopharmaceutical and its interaction with its surrounding tissue.

However, it was not until the 1990s that it has been used as a tool for imaging the physiology and pathology of the brain, and later the heart also. It is now been widely used, in many countries for the detection and staging of a variety of malignant diseases. PET differs from other nuclear medicine procedures, e.g., single photon emission tomography (SPECT), by its ability to detect coincidence signals that allows the quantification of regional tissue radioactivity. It could be achieved by injecting a PET imaging agent or radiopharmaceutical incorporating a positron-emitting radioisotope such as ^{11}C, ^{13}N, ^{15}O, or ^{18}F. These radioisotopes decay to emit a positron that loses sufficient energy to form positronium, and undergoes annihilation to emit two 511 keV gamma rays (more chance). Those positrons that do not form positronium will undergo an alternate process called free electron annihilation. The ability to detect these gamma rays in coincidence in two detectors at 180° is the key to locating the position of the radiopharmaceutical in the body.

Compared with other noninvasive imaging tools, PET has greater sensitivity ($<10^{-8}$ M) and more specificity for its target sites but it lacks the resolution of other noninvasive imaging agents, such as MRI and CT. An advantage of PET is the availability of an array of positron emitting radioisotopes, (with varying half lives and chemistries) which allow for the development of a wide range of PET radiopharmaceuticals for imaging many biological processes. PET unlike CT and MRI, which look at anatomy, studies metabolic activity or body function and scans are used to produce detailed three-dimensional images of the inside of the body. PET tracks a positron emitting radiopharmaceutical injected into the body and generates a three-dimensional image of its location.

In more recent times PET has been used in the risk assessment of new pharmaceuticals. Here the drug is radiolabeled with the positron emitter and the resultant product or probe is used to investigate where the drug (e.g., peptide, protein, or nanoparticles) goes in the body to monitor the uptake, distribution and pharmacokinetics of their drug in vivo. The ability to monitor the product movement noninvasively assists to make more accurate assessment of the drug's therapeutic index and ultimately its effectiveness in the long term. At present, there are great increases in

the application of nanoparticles for molecular imaging that led to the development of both organic and inorganic nanoparticles functionalized to attach with radionuclides, targeting ligands, and polyethylene glycol. PET imaging provides the imaging signal for TDD and provides the information of the pharmacokinetics of the particle. The structure of the nanoparticle allows a broad range of radiolabeled chemistries to be used to attach various PET nuclides to the particle that allows the quantitation and the multifunctionality of nanoparticles to take advantage of this technique.

PET technology is using ^{11}C and ^{18}F radioisotopes for fast track of their distribution inside the body. For larger molecules, (such as peptides, antibodies, DNA and nanoparticles) that have slower clearance rates, a PET isotope with longer half-life is more appropriate. Copper-64 (^{64}Cu) is an emerging PET isotope that has positron energy similar to ^{18}F, but a longer half-life (7 fold) which makes it ideal for attaching to larger target agents (i.e., >1,000 s mol wt). It can be produced in a reactor (using natural copper or enriched zinc targets) and in a cyclotron (using enriched nickel or zinc targets). Unlike ^{11}C and ^{18}F, ^{64}Cu cannot be covalently attached or incorporated into the molecule and therefore needs to be attached through a bifunctional ligand.

Nanocarriers fill a critical position between the macroscopic and molecular level structure and can be designed to offer unique advantages when compared with both macroscopic materials and molecular systems. This characteristic has generated significant interest in synthetic nanomaterials that can serve as vessels for the transport and delivery of imaging and therapeutic agents. Nanocarriers are able to possess internal packaging capacity and a sufficient surface area for presentation of multiple types and numbers of active elements that have an optimal balance of internal volume and external surface area. The promising attributes of nanomaterials for targeted imaging including their ability to deliver large number of imaging agents to achieve high-sensitivity imaging and their ability to deliver several different types of imaging agents to perform multimodality imaging [25, 26]. Figure 21.6 is depicting the ^{18}F-FDG scan for staging evaluation of malignancy, with involvement of multiple lymph nodes in chest and abdomen using PET imaging technique. ^{11}C PET Methionine imaging for evaluation of brain tumor recurrence after radiotherapy as presented in Fig. 21.7 (data not published).

While the range of positron radioisotopes (Table 21.2) generates plenty of opportunity for developing radioactive mimics of drugs, the relatively poor resolution of PET hinders its wider deployment in patient management. The resolution of PET is highly dependent on the energy of the positron, as the annihilation process requires the positron to slow to energies below ~100 eV and form positronium or annihilate with free electrons [27].

21.4.5 Fluorescence Imaging Technique

In the past several years a new concept for super-resolution imaging based on the localization of individual fluorescent molecules has emerged. The use of photoswitchable fluorescent probes allows the overlapping images of many individual

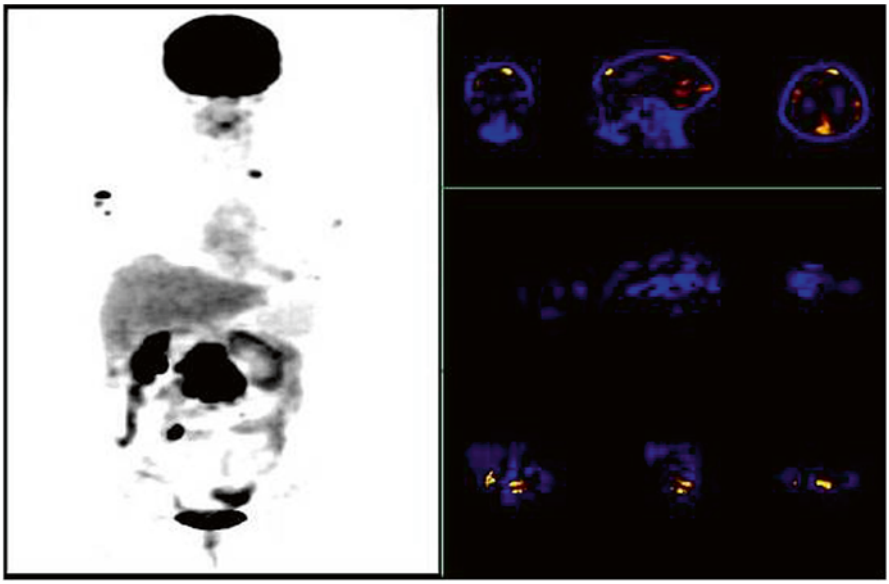

Fig. 21.6 ^{18}F-FDG scan for staging evaluation of malignancy, with involvement of multiple lymph nodes in chest and abdomen

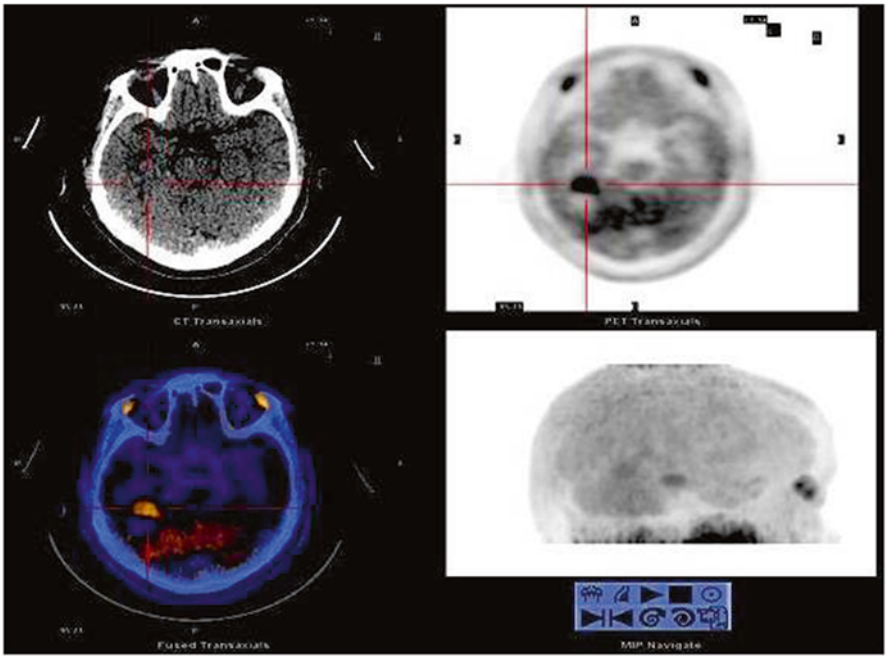

Fig. 21.7 ^{11}C Methionine imaging for evaluation of brain tumor recurrence after radiotherapy

Table 21.2 Selected positron radioisotopes and their physical characteristics

S. No.	Isotope	Half-life	Positron energy (average keV)	Gamma energy and probability (keV; %)
1.	^{82}Rb	72 s	1,535	776.5; 13
2.	^{13}N	9.97 months	10,000	Nil
3.	^{15}O	122.24 s	735	Nil
4.	^{11}C	20.39 min	385	Nil
5.	^{18}F	109.77 min	640	Nil
6.	^{22}Na	2.60 year	545	Nil
7.	^{26}Al	7.4e5y	960	1,808
8.	^{39}K ^{40}K	7.636 min $1.248(3)\times10^{9}$ year	133 341	1,330
9.	^{55}Co	17.53 h	511	1,170
10.	^{68}Ga	67.63 min	836	1,077; 3
11.	^{61}Cu ^{62}Cu ^{64}Cu	12.7 h 9.74 min 12.7 h	278.2 880 1,330	1,345; 1.27
12.	^{63}Zn	38.47 min	3,366.2	3,366
13.	^{70}As ^{71}As ^{74}As	52.6 min 65.28 h 17.77 days	–	1,176
14.	^{82}Rb	1.27 min	3,150	511
15.	^{120}I ^{121}I ^{124}I	88.1 0 months 4.176 days 4.18 days	1,540 (max)	603; 62.9 1,691; 10.88
16.	^{110}In	4.9 h	–	Nil
17.	^{122}Xe	20.1 h	122	Nil

fluorophores to be separated in time and enables precise fluorophore localization on densely labelled samples. The positions of the fluorophores are then plotted to construct a high-resolution image. Fluorescence imaging is used in preclinical and clinical studies that enables fluorescent probe to generate contrast in targeted organ. One of the major advantages of fluorescence microscopy is its capacity for multicolor imaging which allows the relative organization and interactions between different biological structures or molecules to be visualized, e.g., co-localization of differently colored probes. Multicolor imaging requires multiple, optically distinguishable probes. This aspect of fluorescence imaging is essential for probing interactions between biomolecules and acquiring invaluable insights into biological processes owning to its molecular specificity and dynamic imaging capability. However, the spatial resolution of light microscopy, classically limited by diffraction to a few hundred nanometers, is substantially larger than typical molecular length scales in cells. Advantages of the fluorescence imaging are to achieve chemical specificity, single-molecule sensitivity, molecular-scale resolution, and dynamic imaging capability such as molecular interactions inside the cell can be directly visualized. Among the most useful aspects of fluorescence microscopy is its ability to provide a 3D image of the sample [15].

21.4.6 Optical Imaging (OI) Technique

The development of imaging probes is based on advances in nanotechnology. Optical-based techniques can detect early cancerous growth and metastasis in patients. Optical technologies require an exogenous agents (chemical or contrast) and endogenous agents. These agents (e.g., small molecules, peptides, antibodies, viral vectors, nanoparticles, and engineered antibodies) target tissues. To improve sensitivity and specificity of optical imaging, the following areas need to be focused for cancer detection: improvement in processing, probe targeting, probe activation, and use of genetic reporters that are more specific for target location. Endogenous target could be probed by auto fluorescence methods; the major goal is to increase fluorescence intensity. Exogenous contrasts are strategies to identify targets and screen target specific probes. The target might be identified by genomic, proteonomic, physiological (tissue/cellular or subcellular), and/or histological analyses. The selected target can be used in high throughput in vitro screening assays, where thousands of chemical molecules can be screened to identify targets at cellular or subcellular levels based on regulatory interactions with cellular molecules, alterations in cellular functions, etc. It offers a number of important advantages over existing radiological imaging techniques because it employed non-ionizing radiation, which significantly reduces patient radiation exposure and allows for repeated studies over time. It also offers the potential to differentiate among soft tissues, and between native soft tissues and tissue labelled with either endogenous or exogenous contrast media by using their different photon absorption or scattering profiles at different wavelengths. Imaging presents photon absorption and scattering differences and specific tissue contrasts, offers potential capabilities for studying functional and molecular level activities that are the basis for health and disease.

Optical imaging is very amenable to multimodal imaging extends over a wide range on the imaging resolution scale, often complementary to and easily combinable with other imaging techniques. Optical imaging methods are generally least expensive and easiest options for imaging in artificial two-dimensional (2D) porous media, multiphase fluid distribution, fluid flow, solute transport and mixing, colloidal transport, toxicology, deposition, and reactions. Advancements in protein engineering and materials sciences have enabled novel nanoscale targeting approaches that may bring new hope to a number of diseases [16, 24, 28].

The advantages of nanocarrier based targeted drug delivery are as follows:

- Protects the drug from premature degradation.
- Prevents drugs from prematurely interacting with the biological environment.
- Enhances absorption of the drugs into a selected tissue (e.g., solid tumor).
- Controls the pharmacokinetic and drug tissue distribution profile;
- Improves intracellular penetration.

Advancements over conventional drug delivery of optical imaging include:

- To be made from a material that is biocompatible, well characterized, and easily functionalized.

– To exhibit high differential uptake efficiency in the target cells over normal cells (or tissue).
– To be either soluble or colloidal under aqueous conditions for increased effectiveness.
– To have an extended circulating half-life, a low rate of aggregation, and a long shelf life.

21.5 Limitations of the Techniques Studied

TDD getting delivers drugs to their targets in a controlled manner. Imaging techniques based on optical contrast can be used to visualize dynamic and functional properties. Functional imaging techniques other than functional MRI and PET are needed to advance the field of drug delivery. The short half lives and the requirement for specialized skills to incorporate these isotopes have limited the application of PET to small molecules (i.e., <500 mol wt) with fast biological clearance rates. Pure optical imaging, however, has significant drawbacks including poor spatial resolution as a result of the overwhelming scattering of light in biological tissues.

Although the literature suggests that MRI and magnetic resonance angiography (MRA) are accurate and rapid methods but used with increasing frequency, both are associated with a high likelihood of false-positive results [29]. In fluorescence imaging fluorophores lose their ability to fluoresce as they are illuminated (photobleaching). Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence. Photobleaching can severely limit the time over which a sample can be observed by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoprotective scavenger chemicals. Furthermore fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect [30]. In optical imaging technique, surface light passes through different types of tissue, thereby leading to scattering and absorption of light. Therefore, exact spatial localization and quantification of signal intensities are impaired and the bioluminescent image obtained represents only a two-dimensional reflection of the three-dimensional and deeper located signal sources [31].

21.6 Conclusion

Development of suitable screening methodologies for determining optimal characteristics of nanocarriers for targeted drug delivery is the demand of time. The design and optimization of carriers in the way of addressing questions of a particular application, TDD and of keeping them very flexible and reliable for general

applications represent an important step in drug delivery approaches that would ultimately influence the therapeutic efficacy. Furthermore, the systemic therapies using nanocarriers require methods that can overcome nonspecific uptake by mononuclear phagocytic cells/non-targeted cells. Improved therapeutic efficacy of targeted nanocarriers has been well established in different experimental models and more than 100 clinical trials are underway using nanocarrier formulations. The imaging technologies described above could be extensively employed for noninvasive imaging in preclinical and clinical stages. It results in improved therapeutic outcomes and reduced costs.

21.7 References

1. Banerjee PS, Robinson JR (1991) Novel drug delivery systems. Clin Pharm 20(1):1–14
2. Jain S, Dani P, Sharma RK (2009) Pharmacoscintigraphy: a blazing trail for the evaluation of new drugs and delivery systems. Crit Rev Ther Drug Carrier Syst 26(4):373–426
3. Sastry SV, Nyshadham JR, Fix JA (2000) Recent technological advances in oral drug delivery—a review. Pharm Sci Technolo Today 3(4):138–145
4. Sutton D, Nasongkla N, Blanco E, Gao J (2007) Functionalized micellar systems for cancer targeted drug delivery. Pharm Res 24(6):1029–1046
5. Cheng J, Teply BA, Sherifi I, Sung J, Luther G, Gu FX, Farokhzad OC (2007) Formulation of functionalized PLGA–PEG nanoparticles for in vivo targeted drug delivery. Biomaterials 28(5):869–876
6. Langer R (1998) Drug delivery and targeting. Nature 392(6679):5–10
7. Singh R, Lillard JW Jr (2009) Nanoparticle-based targeted drug delivery. Exp Mol Pathol 86(3):215–223
8. Neuberger T, Schöpf B, Hofmann H, Hofmann M, Von Rechenberg B (2005) Superparamagnetic nanoparticles for biomedical applications: possibilities and limitations of a new drug delivery system. J Magn Magn Mater 293(1):483–496
9. Khan MZI, Prebeg Ž, Kurjaković N (1999) A pH-dependent colon targeted oral drug delivery system using methacrylic acid copolymers: I. Manipulation of drug release using Eudragit® L100-55 and Eudragit® S100 combinations. J Control Release 58(2):215–222
10. Dharap SS, Wang Y, Chandna P, Khandare JJ, Qiu B, Gunaseelan S, Minko T (2005) Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide. Proc Natl Acad Sci U S A 102(36):12962–12967
11. Sengupta S, Eavarone D, Capila I, Zhao G, Watson N, Kiziltepe T, Sasisekharan R (2005) Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system. Nature 436(7050):568–572
12. Paciotti GF, Myer L, Weinreich D, Goia D, Pavel N, McLaughlin RE, Tamarkin L (2004) Colloidal gold: a novel nanoparticle vector for tumor directed drug delivery. Drug Deliv 11(3):169–183
13. Wardlaw JM, Chappell FM, Best JJK, Wartolowska K, Berry E (2006) Non-invasive imaging compared with intra-arterial angiography in the diagnosis of symptomatic carotid stenosis: a meta-analysis. Lancet 367(9521):1503–1512
14. Simon BA (2000) Non-invasive imaging of regional lung function using x-ray computed tomography. J Clin Monit Comput 16(5–6):433–442
15. Ntziachristos V, Bremer C, Weissleder R (2003) Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging. Eur Radiol 13(1): 195–208

16. Villringer A, Chance B (1997) Non-invasive optical spectroscopy and imaging of human brain function. Trends Neurosci 20(10):435–442
17. Bhavna, Ahmad FJ, Mittal G, Jain GK, Malhotra G, Khar RK, Bhatnagar A (2009) Nano-salbutamol dry powder inhalation: a new approach for treating broncho-constrictive conditions. Eur J Pharm Biopharm 71:282–291
18. Newman SP, Clark AR, Talalee N, Clarke SW (1989) Pressurised aerosol deposition in the human lung with and without an open spacer. Thorax 44:706–710
19. Ali R, Mittal G, Ali R, Kumar M, Kishan Khar R, Ahmad FJ, Bhatnagar A (2013) Development, characterisation and pharmacoscintigraphic evaluation of nano-fluticasone propionate dry powder inhalation as potential antidote against inhaled toxic gases. J Microencapsul 30(6): 546–558
20. Kumar N, Soni S, Jaimini A, Ahmad FJ, Bhatnagar A, Mittal G (2011) Edetate calcium disodium nanoparticle dry powder inhalation: a novel approach against heavy metal decorporation. Int J Pharm 416:376–383
21. Singh AK, Bhardwaj N, Bhatnagar A (2004) Pharmacoscintigraphy: an unexplored modality in India. Ind J Pharm Sci 66(1):18–25
22. Gabizon A, Martin F (1997) Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Drugs 54(4):15–21
23. Daniel MC, Astruc D (2004) Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chem Rev 104(1):293–346
24. Rechberger W, Hohenau A, Leitner A, Krenn JR, Lamprecht B, Aussenegg FR (2003) Optical properties of two interacting gold nanoparticles. Opt Commun 220(1):137–141
25. Newman JS, Francis IR, Kaminski MS, Wahl RL (1994) Imaging of lymphoma with PET with 2-[F-18]-fluoro-2-deoxy-D-glucose: correlation with CT. Radiology 190(1):111–116
26. Jabour BA, Choi Y, Hoh CK, Rege SD, Soong JC, Lufkin RB, Bailet J (1993) Extracranial head and neck: PET imaging with 2-[F-18] fluoro-2-deoxy-D-glucose and MR imaging correlation. Radiology 186(1):27–35
27. Smith SV (2009) Positrons as imaging agents and probes in nanotechnology. J Phys Conf Ser 2009:012044. doi:10.1088/1742-6596/185/1/012044
28. Grinvald A, Bonhoeffer T, Vanzetta I et al (2004) High-resolution functional optical imaging: from the neocortex to the eye. Ophthalmol Clin North Am 17(1):53–67
29. Schwab KE, Gailloud P, Wyse G, Tamargo RJ (2008) Limitations of magnetic resonance imaging and magnetic resonance angiography in the diagnosis of intracranial aneurysms. Neurosurgery 63(1):29–34
30. Gunkel M, Erdel F, Rippe K, Lemmer P, Kaufmann R, Hörmann C, Amberger R, Cremer C (2009) Dual color localization microscopy of cellular nanostructures. Biotechnol J 4(6): 927–938
31. Balas C (2009) Review of biomedical optical imaging—a powerful, non-invasive, non-ionizing technology for improving in vivo diagnosis. Meas Sci Technol 20:104020. doi:10.1088/0957-0233/20/10/104020

Part VIII
Nanotoxicology and Regulatory Issues

Chapter 22
Evaluation of Lung Toxicity of Biodegradable Nanoparticles

Nadège Grabowski, Hervé Hillaireau, Juliette Vergnaud, and Elias Fattal

Abbreviations

7-AAD	7-Aminoactinomycin D
BAL	Bronchoalveolar lavages
BALF	Bronchoalveolar lavage fluids
BALT	Bronchial associated lymphoid tissue
CLSM	Confocal laser scanning microscopy
DPI	Dry powder inhaler
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
ELISA	Enzyme linked immunosorbent assay
IL	Interleukin
IPL	Isolated perfused lung
LDH	Lactate dehydrogenase
MDI	Metered dose inhaler
PEG	Polyethylene glycol
PLA	Poly(lactic acid)
PLGA	Poly(lactide-co-glycolide)
PMN	Polymorphonuclear
PVA	Polyvinyl alcohol
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

N. Grabowski • H. Hillaireau • J. Vergnaud • E. Fattal (✉)
Faculty of Pharmacy, Institut Galien Paris-Sud, University of Paris-Sud, Châtenay-Malabry, France
e-mail: elias.fattal@u-psud.fr

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_22

SLN	Solid lipid nanoparticles
SP	Surfactant proteins
TEM	Transmission electronic microscopy
TNF-α	Tumor necrosis factor-α

22.1 Introduction

The term "nanotoxicology" was introduced in 2004 by Donaldson and coworkers [1] and defined as "a new frontier in particle toxicology relevant to both the workspace and general environment and to consumer safety." The concept was aiming to create a new subdiscipline in toxicology, meeting specific aspects of nanoparticle toxicity and modifying the existing tests to adapt them to the evaluation of risks associated to nanoparticles (Fig. 22.1).

Nanoparticles are nowadays being used in a large variety of manufactured products, such as electronic components, textile, or cosmetics [2]. In addition, very promising applications in medicine have been highlighted in diagnostic and therapeutic. For such purposes, nanoparticles can enter the organism using different routes such as inhalation, oral ingestion, dermal or parenteral route. Despite a high number of promises for therapeutics and diagnosis, nanoparticles are potentially able for each of these routes of administration to induce adverse effects and toxicity. Among all the tissues susceptible to be affected by nanoparticle interaction are the lungs which have been widely explored, mostly for inorganic nanoparticles [3]. A large number of in vitro and in vivo studies have reported cytotoxic effects, inflammatory and oxidative stress responses from lung cells after exposure to particulate matter, silica [4], titanium dioxide [5, 6], gold and silver [7] nanoparticles. The involuntary inhalation of these materials through accidental exposure therefore represents a potential high source of toxicity. Nevertheless, the pulmonary route is also an interesting noninvasive route of administration for drug delivery [8]. Both local and systemic treatments can be considered taking advantage of the lungs' large absorption surface area, and high vascular permeability with continuous exchanges between alveoli and blood [9]. In addition, the weak enzymatic activity present in lungs is relevant for administration of poorly stable drugs (peptides/proteins). Administration of drug loaded nanoparticles can lead to several advantages. Ideally, nanoparticles will not only protect drug against degradation and transport it to target cells, but also allow a sustained drug release, decreasing the frequency of administration and subsequent risk of side effects [10]. Nanomedicine can be delivered to the lungs by nebulization of an aqueous nanoparticle suspension or aerosolization of a dry powder of nanoparticles [11].

A large variety of nanoparticles made of polymers or lipids can be used to prepare nanomedicines [12]. Most of them have been investigated for lung delivery of antibiotics, anti-inflammatory, anticancer drugs, or hormones.

The question of whether nanomedicines are safe remains debated. For this purpose, toxicity studies should be carried out in vitro, in vivo or ex vivo after exposure to nanomedicine. Toxicity encompasses different mechanisms, from cellular

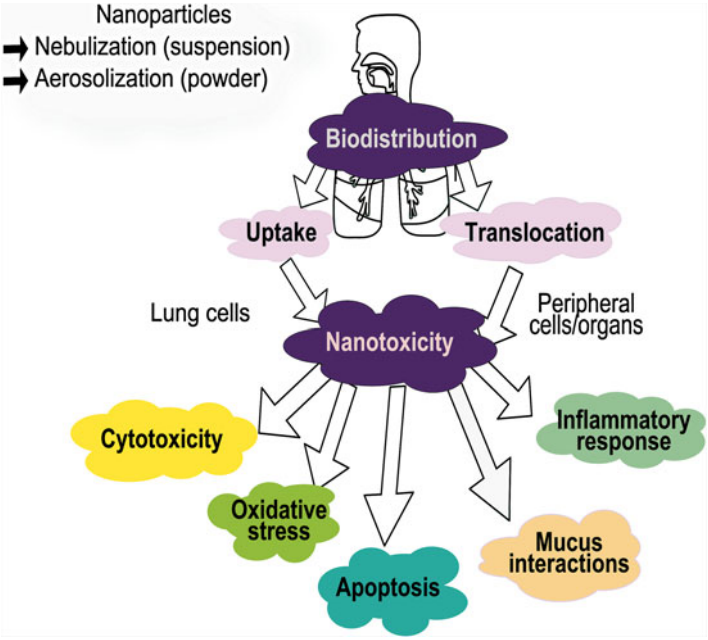

Fig. 22.1 General roadmap to assess lung toxicity of nanoparticles

viability, to inflammatory response, oxidative stress induction, and DNA damage that should be deeply investigated.

In the present chapter, the pulmonary tract architecture and its defense barriers are firstly presented. Secondly, the nanoparticles used for lung delivery, and their administration modes are detailed. The toxicity tests used to investigate toxicity of nanoparticles and the variety of pulmonary tract models developed so far are then illustrated by their application for the evaluation of nanoparticle toxicity.

22.2 Structure of the Respiratory Tract

22.2.1 Anatomy

The respiratory tree is in charge of bringing oxygen to the body and removing carbon dioxide through continuous gas exchanges with blood circulation. The lung volume is included in the range of 2.5 (children) to 7 l (adults) depending on the lung size [13]. According to the model proposed by Weibel [13], the respiratory tree is a hierarchical and symmetric network, divided into 24 generations, beginning with the trachea (generation 0) and ending with alveoli (generation 23). Each airway is divided into two airways increasing the specific surface, to carry the air up to alveolar ducts where gas exchanges occur. During each breath, inhaled air is

transported to the conductive zone (generations 0–4), including the trachea and bronchus that divides into two distal bronchi (dichotomy), in order to reach the respiratory zone. As reviewed by Shelly et al. [14], the temperature and the moisture of alveolar gases are essential parameters in normal lungs to maintain physiological conditions. Conditions of optimal inhaled air were determined as 100 % of humidity, and a temperature closed to the body [15], modulated by the nose and the upper respiratory tract.

While the conductive zone represents 10 % of the lung volume, the respiratory zone represents the lasting 90 % [13]. The respiratory zone is composed of 3–500 million alveoli and 14 million alveolar ducts. The average alveolar diameter in human lungs is in the range of 250–290 μm, among the lung volume. With an average diameter of 400–450 μm, 3–4 generations of alveolar ducts are terminating in alveolar sacs that have exactly the same structure. In addition, three generations of respiratory bronchioles, with an average diameter of 500 μm, are proximal to alveolar ducts. Blood vessels follow exactly the same pattern than the bronchial tree, with similar dimensions. The pulmonary arteries divide into 28 generations, with an average diameter of 15–25 μm. In the respiratory zone, 277 billions of capillary segments, with an average diameter of 8.3 μm create a total tissue–blood interface (or alveolar–capillary surface) of 60–80 m^2 for the whole lungs.

22.2.2 Histology

The bronchial epithelium is pseudostratified and is composed of three cell types: basal cells, ciliated cells (allowing mucus elimination), and goblet cells (secreting mucus) (Fig. 22.2). The epithelium thickness is close to 60 μm and covered with 2–10 μm of an airway fluid [16, 17]. The latter is composed of two layers cleared by ciliary beating, the mucus and the periciliary fluid (consisting of water layer that cover cilia cells on apical surface) separated by a surfactant layer [18]. Mucus from healthy human lungs is nonhomogeneous and viscous (rheology of a cross-linked gel) with a pH ranging from 7 to 8.5. Biochemical analyses have shown that human mucus samples are mostly made of water, containing 7 % solids, 8–20 % mucins, and less than 1 % DNA. Secreted mucins (glycoproteins) form a 3-dimensional polymer meshwork able to trap particles [17], with pores from 10 to 100 nm [19]. Mucins are made of 70–80 % carbohydrates, 20 % proteins, and 1–2 % sulfates. Eight proteins encoded as MUC are present in mucus layer, with a large majority of MUC5AC (~80 %) and MUC5B (~20 %) [17] expressed by goblet cells and glandular mucous cells respectively [20]. In terminal bronchi, the epithelium has a cuboid shape with a thickness of 10 μm, is covered with 3 μm of airway fluid and is composed of ciliated and Clara cells (producing mucus, pulmonary surfactant proteins and cytokines, and involved in epithelium regeneration) [16].

Alveolar type I cells (8 %) involved in gas exchanges coexist with alveolar macrophages (3–5 %, up to 19 % in smoker subjects), alveolar type II cells (16 %, cover 7 % of the alveolar surface), capillary endothelial cells (30 %) and cells in the interstitial spaces (37 %) [21, 22] (Fig. 22.2). The alveolar macrophages perform phago-

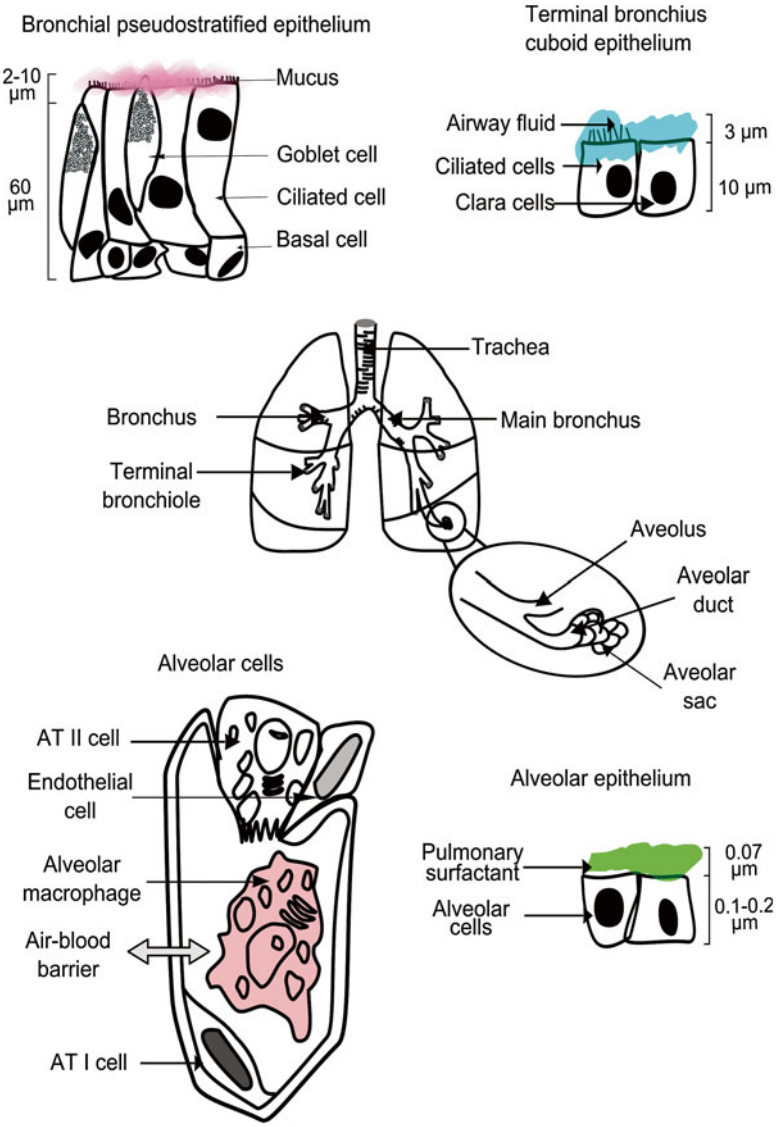

Fig. 22.2 Structure of the respiratory tract and cellular composition of human lung bronchial/terminal bronchial and alveolar epithelia (*ATI/II* alveolar type I/II cells)

cytosis of foreign particles or pathogens. The endothelial cells, lining lung blood vessels, are highly metabolic and are involved in the circulation of bioactive substances such as peptides, lipids, and prostaglandins. Alveolar type II cells produce lung surfactant components and are involved in the regeneration of type I pneumocyte cells by differentiation. In alveolar ducts, the thickness of the epithelium is around 0.1–0.2 µm and covered with 0.07 µm of pulmonary surfactant [16]. The pulmonary surfactant is composed of lipoproteins, 90 % of which are phospholipids

and 10 % are surfactant proteins (SP), secreted by type II alveolar cells [22]: SP-A (the most abundant), SP-B, SP-C, SP-D. SP-B and SP-C, very small and hydrophobic proteins, reduce the surface tension at the air–liquid interface of the lung, that is essential to avoid collapse. SP-A and SP-C, members of collectin family, participate to the innate immunity, before induction of an antibody-mediated response [22, 23]. SP-A and SP-C, present also in non-pulmonary sites, are involved in viruses and bacteria uptake by phagocytes [24]. SP-A was also shown to be involved in the uptake of nanoparticles covered with different polymers [25].

22.3 Clearance Mechanisms in Lungs

Defense mechanisms are both immunological and physiological. The Bronchial Associated Lymphoid Tissue (BALT) plays a major role in the immune response to all kinds of antigens, with a local response faster and more protective than those initiated by the systemic system [26]. Absent in the lungs of healthy newborn babies, the BALT starts its development after meeting the first antigens. Generally located along the bronchial airways and the airway bifurcations, the BALT contains essentially B lymphocytes, dendritic cells, macrophages, T lymphocytes, and M cells. M cells present the antigen to dendritic cells that participate in maintaining the alert BALT.

A major clearance mechanism is also achieved physiologically by the mucociliary escalator. Between 2 and 24 h after inhalation, most of the foreign particles are expectorated, with a rate closed to 5 mm/min in peripheral zone and 20 mm/min in trachea [27]. The second clearance pathway consists in phagocytosis performed by resident alveolar macrophages. Particles are then enzymatically degraded or driven out by the mucociliary clearance, or brought to trachea-bronchial lymphatic canals by translocation. Phagocytosis is modulated, not only by particle size, but also by particle geometry [28]. Some inhaled particles can be metabolized thanks to detoxification enzymes, such as cytochrome P450, but compared to other organs such as the liver, this pathway is negligible [29].

One other barrier is the size of the inhaled material [30]. For lung delivery, the geometric diameter, particle shape, and density are taken into account resulting in the so-called aerodynamic diameter. The effective aerodynamic diameter of the particles affects the magnitude of forces acting on them. While inertial and gravitational effects increase with increasing particle size, diffusion produces larger displacement as particle size decreases. Large particles (>5 μm aerodynamic diameter) usually impact on airway wall at bifurcations. They usually deposit higher up in the airway such as the back of the throat or pharynx [31]. When the aerodynamic parameter of particles ranges from 1 to 5 μm, they undergo sedimentation by gravitational force that occurs in smaller airways and respiratory bronchioles. For small particles (<1 μm aerodynamic diameter), their movements are controlled by Brownian motion. The optimal particle size for efficient deposition at the lower respiratory tract is considered to be between 1 and 3 μm [11, 32]. As the particle size

further decreases, deposition in the lung increases again due to the increasing mobility through diffusion [30]. However, only nanoparticles that are less than 100 nm, appear to settle effectively to the alveolar region with a fractional deposition of around 50 % [16, 33]. When the diameter gets larger in the nanoscale range, a high proportion, up to 80 %, can be exhaled [34, 35].

22.4 Nanoparticles for Drug Delivery

22.4.1 Nanoparticles and Lung Delivery

Nanoparticles have been extensively used for lung drug delivery for the administration of a large variety of drugs (antibiotic, anticancer, anti-inflammatory, peptide, protein, or nucleic acid drugs) that have been loaded into lipid or polymeric nanoparticles (Fig. 22.3) (for review see [10, 12, 36]). Nanoparticles can induce both local and systemic delivery after release of the encapsulated drug. Major successes were obtained with lipid or polymer nanoparticles. For instance, unilamellar or multilamellar liposomes ranging from 100 nm to 1 μm made from the self organization of glycerophospholipids or non-ionic surfactants were produced for lung delivery. One of their major advantages for pulmonary administration is their biocompatibility due to their natural origin and their lipid composition quite often close to the one of the lung surfactant. Liposomes were used to deliver peptides and proteins to the

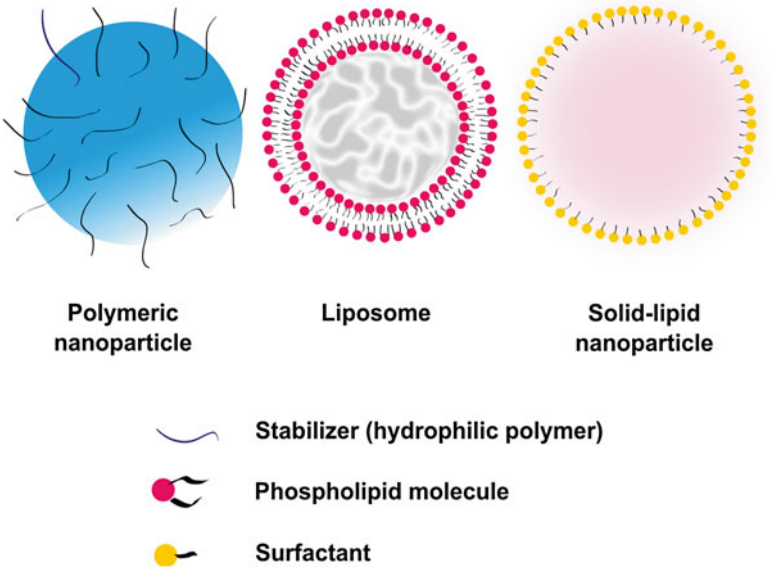

Fig. 22.3 Nanoparticles used for lung drug delivery

lungs [37] and were already investigated in phase II clinical trials to deliver amikacin [38] or ciprofloxacin [39]. Furthermore, several studies investigated the potential of solid lipid nanoparticles (SLN) composed of a solid lipid core (dispersed in water) and a shell of stabilizers (soy lecithin or poloxamer) [40]. SLN have shown a great potential for insulin delivery towards lungs [41].

Last but not least, polymeric nanoparticles are of great interest for lung drug delivery [42] since they can be tailored from a range of biodegradable polymers, such as poly(lactic acid) (PLA), poly(lactide-co-glycolide) (PLGA), chitosan [43], gelatin or alginate [44]. Stabilizers like polyvinyl alcohol (PVA), chitosan, or poloxamers are often required, in order to obtain spheres in the size range of 100–250 nm. However, their presence at the surface can modify cell–nanoparticle interactions and should be carefully investigated. For instance, a large amount of PVA significantly decreases cellular uptake [45] or the presence of chitosan modulates dendritic cell recognition [46]. Other surface modification such as PEGylation (using polyethylene glycol (PEG)) of polymeric nanoparticles has been shown to decrease the time of mucus layer crossing [19].

22.4.2 Administration Modes in Lung Delivery

Nanomedicines can be delivered to the lungs by two modes, under liquid (using nebulizers or Metered Dose Inhalers—MDIs) or solid form (using Dry Powder Inhalers—DPIs). Knowing the administration mode is crucial before conducting a nanotoxicity study, since nanoparticle biodistribution will be different according to their mode of entry, which might impact the type of cells that will be first in contact with nanoparticles.

22.4.2.1 Delivery Under Liquid Form

Nebulization is the easiest administration mode to deliver aqueous suspensions of nanoparticles or liposomes around 200 nm [47], but it is not convenient for the delivery of large particles (0.3–3 μm) that can provoke nasal obstructions or be filtered by the nasal mucosa [48]. To achieve nebulization, two devices are available: nebulizers (delivery under controlled rate) and MDIs. The MDI is a pressured device that allows the delivery of uniformed doses of suspension/solution to the patient [49]. Droplets are formed under pressure and further inhaled by the patient. MDI were originally developed to deliver anti-asthmatic treatments. Although there are a small number of applications of MDI for the delivery of nanoparticles, they have demonstrated an interest for the delivery of chitosan nanoparticles to the deep lung [50]. On the contrary, nebulization is the preferred mode that in most cases is achieved in vivo in small animals (rats and mice) with a specific device, for example the MicroSpray™ aerosolizer (Penn-Century for liquid) which was first used to deliver an aerosol of fluorescent nanoparticles to mice [51]. No mortality was induced by the technique, and one day after administration, the mice showed full recovery [51].

22.4.2.2 Delivery Under Solid Form

Reproducible doses of solid particles can be delivered using DPI [52]. A special veterinary device is available, called Dry Powder Insufflator™ Pulmonary Aerosol Kit (Penn-Century for solid) for in vivo studies in small animals [53]. However, solid nanoparticles are never used as such in DPI because they are too small to be delivered in a great amount in deep lung regions (i.e., alveolar ducts), most of them being exhaled. To overcome this constraint, different methods have been employed to deliver nanoparticles micro-aggregates that turn into the native nanoparticles once reaching the target (Fig. 22.4). Tsapis et al. have proposed to spray-dry nanoparticles into controlled micro-aggregates, called Trojan microparticles [11]. Trojan particles have several advantages, such as reduction of the delivered dose, increasing the drug bioavailability, controlling the release, reducing the toxicity, and as a result improving the therapeutic index and patient compliance [54]. In these "aggregates," insulin was loaded into chitosan nanoparticles [55] whereas rifampicin [56], TAS-103 (anticancer drug) [57], or dexamethasone [58] were associated to PVA-coated PLGA nanoparticles. The nanoparticles were spray dried in the presence of trehalose [56, 57] or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and hyaluronic acid [58] forming microparticles ranging from 2 to 5 μm. For such aerodynamic diameter values, the deposition in deep lung is favored [55]. The efficacy of the decomposition of microparticles into nanoparticles in contact with water is dependent on the spray-dried optimal temperature and the ratio of primary nanoparticles [56]. The size of microparticles is in addition dependent on the diameter of primary nanoparticles. Conversely, when the spray-drying process is performed at optimal temperature, the fine particle fraction is 20–30 times higher than those of primary nanoparticles, and is almost not affected by the ratio and the size of primary nanoparticles [57]. However, compared to primary nanoparticles, the drug was released more rapidly. Indeed, during the spray-drying process, samples can become amorphous, a phenomenon which accelerates moisture absorption and thus the drug release [59]. Shi et al. have formed PLGA nanoparticle flocs [60]. In this process, PLGA nanoparticles were pre-formulated by emulsion/solvent extraction with two oppositely charged polyelectrolytes (PVA vs. poly(ethylene-maleic anhydride)) and then mixed in water under controlled rate. Following electrostatic interactions, flocs were formed with a large superstructure and were then freeze-dried to obtain a dried powder formulation. To be delivered in deep lungs, tobramycin was loaded in pre-formulated PLGA nanoparticles embedded into microparticles after a bath in lactose aqueous solution [42]. Microparticles were finally freeze dried to be intratracheally delivered to rats. Last, but not least, the use of large porous effervescent microparticles to deliver ciprofloxacin [61] or doxorubicin [62, 63] to the lung was reported. Primary drug-loaded polymeric nanoparticles (polycyanoacrylate, PLGA) were spray dried with, among other excipients, lactose (charge), ammonia (to ensure pH to 8) that were mixed to citric acid and sodium carbonate that cause effervescence in contact with water. Results have shown that the shape, the size, the density and the fine particle fraction of microparticles are mediated by amounts of lactose, PEG, leucine [61] and bicarbonate [64]. Moreover, thanks to the effervescence of the mixture, the release rate of the ciprofloxacin was demonstrated higher than with lactose microparticles.

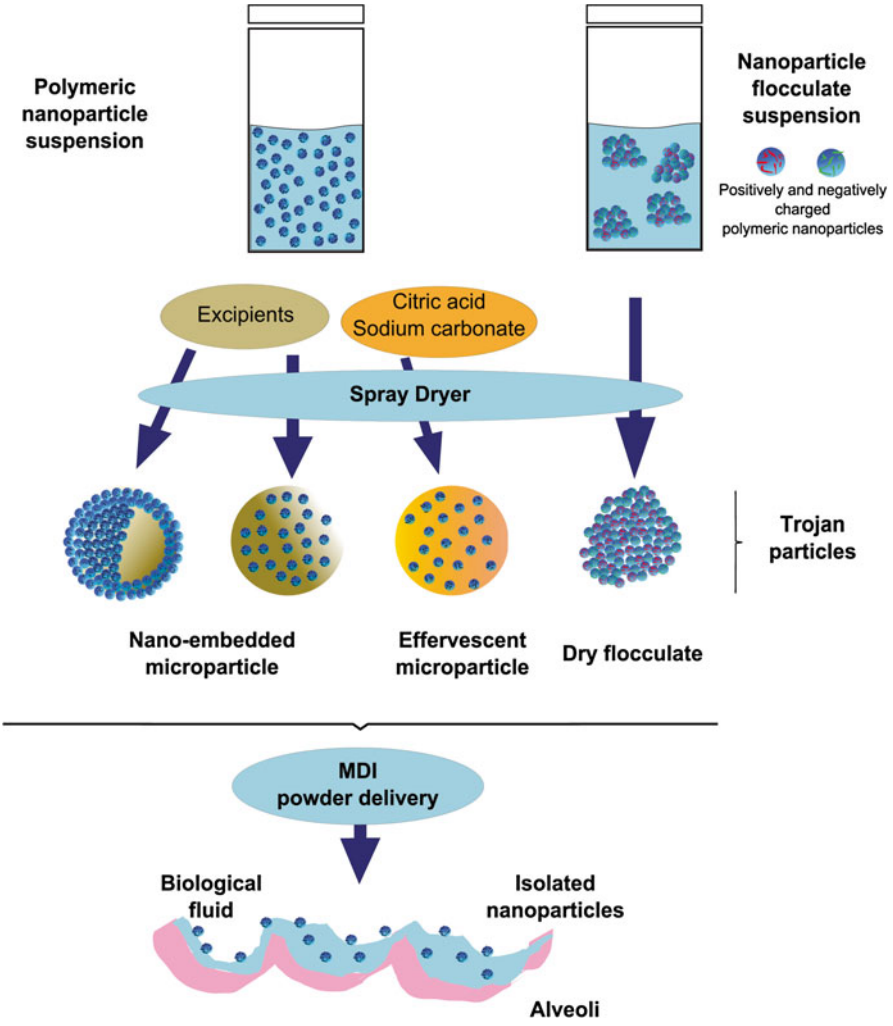

Fig. 22.4 Modified nanoparticles for an aerosol delivery. To form nano-embedded microparticles, the mainly used excipient is lactose, but the use of mannitol, gum arabic, 1,2-dipalmitoylphosphat idylcholine (DPPC), hyaluronic acid, polyvinyl alcohol, or whey protein is also reported (for review see [54])

Recently, several in vitro studies have proposed to spray powders within cell cultures using as receptor the cascade impactor, described in the European pharmacopoeia, which is designed to mimic the respiratory tract. Under pressure, a breath is simulated. Bronchial or epithelial cells have been cultivated on Transwell® at the air–liquid interface and, once the confluence was reached, they were incorporated to the impactor system and exposed to dry powder. The complex system allowed studying the effect of active drugs [65] or diesel particles [66] that reached cell cultures by impaction.

22.4.3 Fate of Nanomedicine After Lung Delivery

22.4.3.1 In Vivo Biodistribution

Biodistribution studies have been performed after nanoparticle delivery in several animal species (mainly mice and rats), and tissue distribution has been limited to the pulmonary regions or extended to peripheral organs. Fluorescent or radiolabeled nanoparticles have been mostly utilized for this purpose. Ungaro et al. [42] have administered to rats, through the Dry Insufflator device™, spray-dried PLGA nanoembedded microparticles (in lactose) containing rhodamine. After administration, the animals were anesthetized, the abdominal cavity incised and lung infused, firstly with phosphate buffer and then with formaldehyde. Respiratory tissues were removed, cut into thin slices to be observed and analyzed by fluorescence microscopy [67]. The results have demonstrated that in vivo nanoparticle distribution was mediated by surface covering [42]. Indeed, the microparticles were found in trachea, bronchia, and bronchioles when the primary nanoparticles were stabilized with PVA, whereas the microparticles were mainly found in alveolar ducts when the primary nanoparticles were stabilized by chitosan (Fig. 22.5).

Other biodistribution studies have shown that after lung delivery into healthy or tumor-bearing mice, nanoparticle fate differs. Indeed, when fluorescent gelatin nanoparticles were delivered by nebulization, healthy mice rapidly eliminated nanoparticles towards kidneys, whereas tumor-bearing mice retained them in lungs, until 24 h [68]. Moreover, 30 min after administration, most of the nanoparticles were found in association with epithelial cells lining the trachea. Gelatin nanoparticles were also indentified in blood vessels, heart, spleen, brain and liver. After aerosolization of effervescent microparticles [62, 63] containing radiolabeled nanoparticles loaded with doxorubicin, the latter were disseminated in the lung whereas the heart, a tissue sensitive to the toxicity of doxorubicin, remained free of nanoparticles.

Finally, free or SLN-loaded radiolabeled amikacin (170 nm, spherical) was delivered to rats by nebulization or intravenous (iv) administration [69]. While after lung delivery of SLN, sustained concentrations were obtained in the lungs, with all other conditions, the rate of amikacin reaches a maximum 30 min following the delivery, after which it started to be cleared. Moreover, 6 h after delivery of SLN by pulmonary route, a significant amount of amikacin was found in the stomach, most likely because the animal swallowed exhaled SLN. As far as the amikacin rate in blood is concerned, the delivery of SLN, either by pulmonary route or iv administration, led to lower drug concentration than after the administration of free drug.

22.4.3.2 Translocation

It is suggested that a small fraction of nanoparticles that are not trapped in alveolar macrophages or in lung epithelial cells have the potential to cross the lung blood–air barrier to reach extrapulmonary spaces and blood circulation [70]. Kunzli and Tager [71] have reviewed the impact of inhaled air nanoparticles on the cardiovascular system.

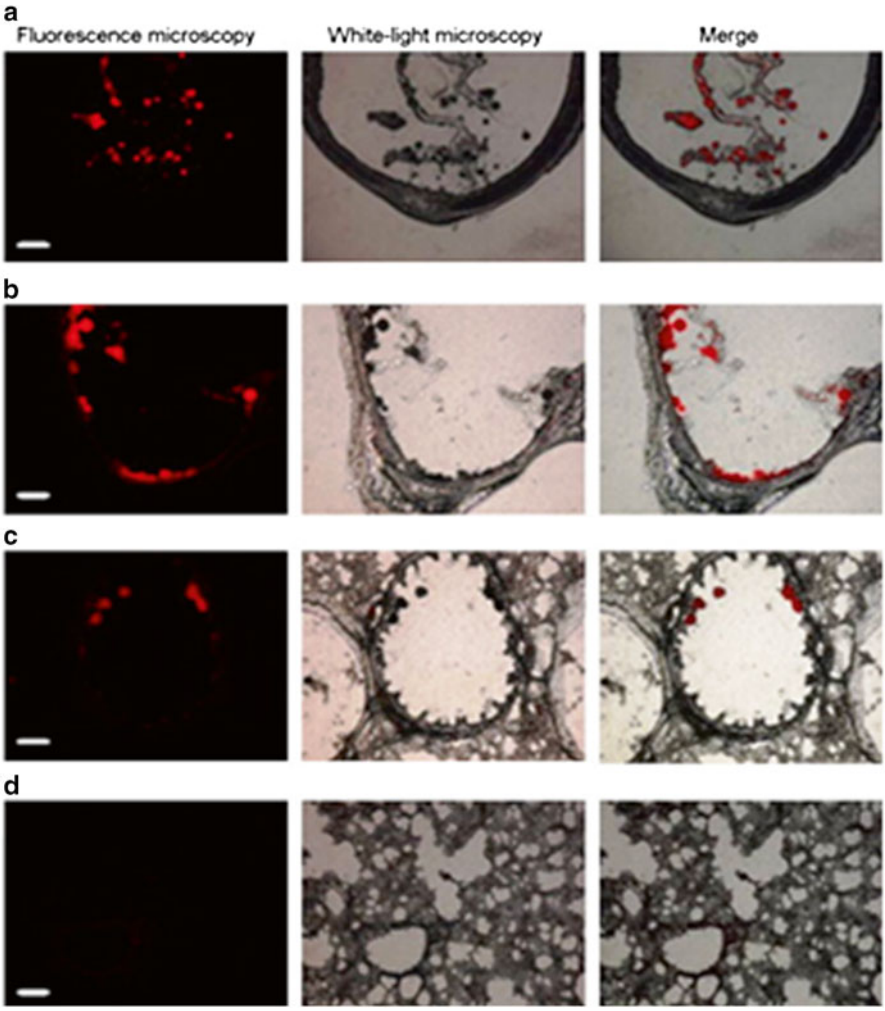

Fig. 22.5 In vivo biodistribution of Rhodamine-PLGA nano-embedded microparticles in rat lungs. Photomicrographs show localization of fluorescent nanoparticles (*red*) in section of trachea (**a**), left primary bronchus (**b**), lobar bronchus (**c**), and alveolar ducts (**d**). (Modified from [67])

Adverse effects, such as heart rate, blood pressure, or inflammatory state, were attributed to nanoparticle translocation from lung to the blood circulation. An in vivo comparative study including inorganic (silica, quantum dots)/organic (polystyrene, human serum albumin) hybrid nanoparticles have demonstrated that translocation is size- and surface-dependent, whereas the composition of the core (organic/inorganic) does not have any influence [72]. Regarding the size, it was shown that below 34 nm, nanoparticles rapidly translocate from the alveolar space to the lymph nodes, followed by a second translocation towards the bloodstream. Secondly, zwitterionic, anionic and polar

nanoparticles are able to translocate, whereas cationic nanoparticles are probably trapped by epithelial cells or macrophages which slow down the translocation. Finally, zwitterionic nanoparticles with an aerodynamic diameter under 6 nm can be found in lymph nodes within 3 min after exposure. After 30 min they start accumulating in the kidney before being excreted [72].

22.4.3.3 Mechanisms of Cellular Uptake In Vitro and In Vivo

Cellular uptake results from the ability of nanoparticles to cross cell barriers. Nanomedicines are generally taken up by cells following endocytosis mechanisms, either phagocytosis or pinocytosis. Phagocytosis is generally performed by phagocytic cells such as macrophages, whereas pinocytosis is performed by all kind of cells. Pinocytosis mechanisms can be clathrin-dependent endocytosis or clathrin-independent endocytosis. This last pathway is classified as caveolae-dependent endocytosis, clathrin and caveolae-independent endocytosis, and macropinocytosis [28, 73]. To address the question of uptake by endocytosis, a simple method consists in performing uptake studies simultaneously at 37 and 4 °C (Fig. 22.6). A nanoparticle uptake at 4 °C similar to the one at 37 °C characterizes a non-energy dependent mechanism such as adsorption, whereas differences of uptake results from endocytosis. To further determine the endocytosis pathways, various chemical (amiloride, chlorpromazine, genistein, or filipin) or biological (AP180 or caveolins) inhibitors can be used to block one or more pathways [75]. For instance, genistein can inhibit several tyrosine kinases that inhibit caveolae pinching, or caveolin can stabilize receptors in caveolae.

In order to visualize nanoparticle trafficking within cells and their exact location, two microscopic modes may be used, which are confocal laser scanning microscopy (CLSM) as well as transmission electronic microscopy (TEM). In the first case, fluorescent nanoparticles are required. For that reason, polymeric nanoparticles can be loaded with fluorescent compounds, such as 6-coumarin [76] or for ensuring greater stability, covalently labeled with rhodamine [77], fluorescein derivates [43], or near infrared emitting fluorescent probe [78]. For more accuracy and complete details, each cell compartment, i.e., endoplasmic reticulum, endosome, Golgi apparatus, lysosome, mitochondria, nucleus, or plasma membrane, can be labeled with specific commercial fluorophores (Cell Staining Simulation Tool Life Technologies [79]). In the case of transmission electronic microscopy, nanoparticles must be electron dense. Otherwise, it is difficult to discriminate between nanoparticles and cell vesicles. For this purpose, in one study, polymeric nanoparticles have been loaded with osmium tetraoxide, to be detectable [76].

The quantification of the in vitro [80] cellular uptake is performed by flow cytometry but some studies use radioactivity sampling or fluorescence intensity measurements [53, 81].

Nanoparticle cellular fate after pulmonary delivery is a crucial point to be investigated when carrying out toxicity analyses. Indeed, the same type of nanoparticle

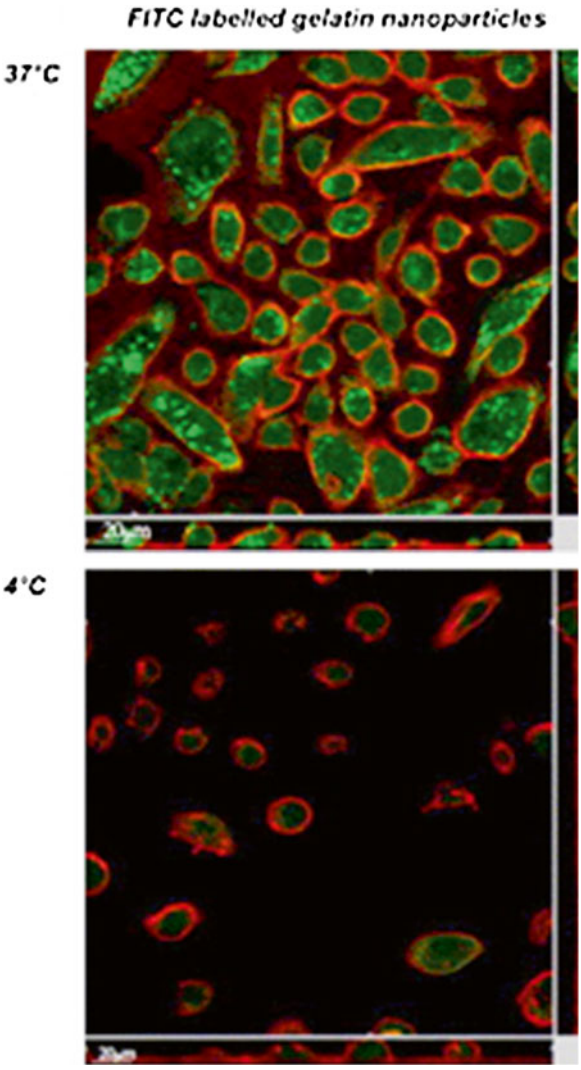

Fig. 22.6 Confocal microscopy imaging (section showing interior of cells) of primary pulmonary epithelium cells after exposure to gelatin nanoparticles at 37 and 4 °C. The cell membrane is bounded with TRITC-concanavalin is coded red and nanoparticle of gelatin bound with FITC is coded green, at 37 and 4 °C. (Modified from [74])

can be taken up by all the previously described ports of entry. Clearance of radioactive-labeled ultrafine nanoparticles was followed after rat intratracheal intubation [82, 83]. The nanoparticles were mostly retained in lungs up to 6 months after exposure, and the clearance was mainly performed by excretion. The small fraction of free nanoparticles found in bronchoalveolar lavages is mostly associated with alveolar or epithelial cells. Translocation into the circulation and accumulation in other organs were also reported, but nanoparticle levels in the extrapulmonary spaces quickly decreased within 7 days [82]. According to the size, nanoparticles were either retained by epithelium or sequestered by alveolar macrophages.

22.5 Toxicity Endpoints

The term toxicity is very large and embraces several parameters (Fig. 22.7). This section provides an overview of the different in vitro, in vivo or ex vivo tests reported in the literature to study nanoparticle-related toxicity.

22.5.1 Cell Integrity

Cell integrity can routinely be estimated by trypan blue, eosin or acridine orange which are stains that selectively color damaged cells [84]. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, or equivalents [MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), WST-1 (Water Soluble Tetrazolium salts)] [85] are

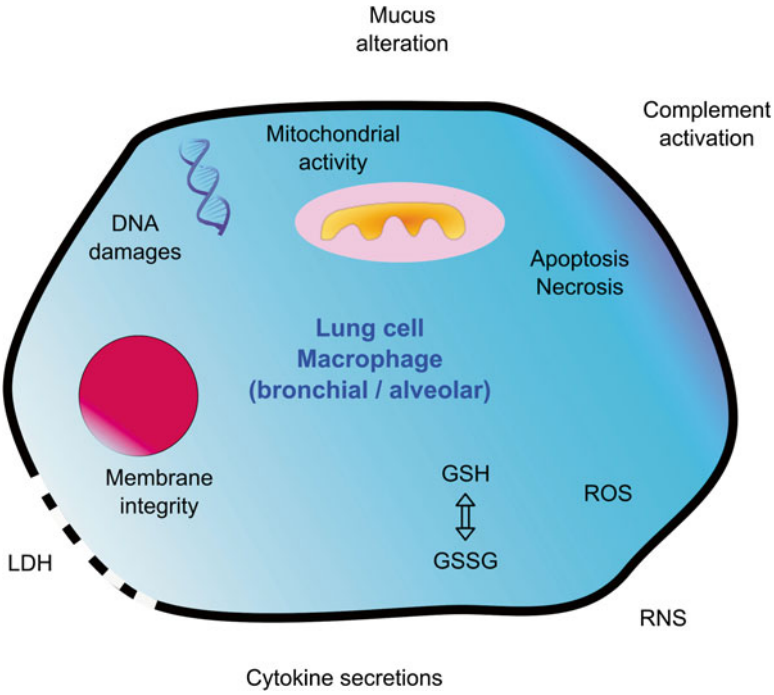

Fig. 22.7 Nanotoxicology can be expressed by several endpoints: evaluation of mitochondrial activity evolution, membrane integrity, inflammatory response, oxidative stress (apoptosis/necrosis detection, analyses of the complement activation, and DNA damage). Tests are performed in supernatants, or inside lung cells (*GSH/GSSG* glutathione sulfhydryl/oxidized disulfide forms, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *SOD* superoxide dismutase)

widely used to assess the in vitro metabolic activity of cells. In these tests, the tetrazolium dye is reduced to formazan purple crystals in living cells by Nicotinamide Adenine Dinucleotide Phosphate-Oxidase-(NADPH) dependent oxidoreductase enzymes. The measurement of the absorbance of the colored solution is directly related to the cellular metabolic activity.

Membrane integrity can also be evaluated by different methods. One of the most commonly used tests is the lactate dehydrogenase (LDH) release assay, suitable for both in vitro and in vivo models [86]. When cell membrane gets disrupted, the intracellular LDH is released in extracellular medium. The released LDH reduces NAD+ into NADH which catalyzes the reduction of a tetrazolium salt to form a formazan crystal with an absorbance wavelength of 540 nm. By the use of a different principle, the Live/dead® test—or equivalent—also allows investigating membrane integrity. To achieve these tests, cells are collected and put in contact with two fluorescent probes able to link nucleic acids, (1) one red-orange, generally propidium iodide (or 7-Aminoactinomycin D (7-AAD)), an intercalating agent, able to penetrate the cells with damaged membrane, and (2) one green, such as the acetomethoxy derivate of calcein or Syto® 24, able to penetrate all the cells. The cells can then be observed by microscopy, or the intracellular fluorescent level can be determined by flow cytometry.

22.5.2 Apoptosis

Apoptosis corresponding to programmed cell death is generally opposed to necrosis that is "accidental" cell death [87]. In apoptotic cells, membrane phospholipids undergo a "flip-flop" event in which phosphatidylserine molecules are translocated to the outer leaflet, to be recognized by macrophages, without disruption of the membrane in the early stages [88], whereas the necrotic cell membrane is disrupted. Quantification of apoptotic cells can be performed by detecting caspase-3, an activated protease in apoptotic cells [89], or by flow cytometry after staining of phosphatidylserines with annexin V, an anticoagulant [90]. To discriminate apoptotic cells from necrotic and intact cells, annexin V, coupled with FITC (green fluorescence) is combined with propidium iodide (or 7-AAD) (red fluorescence) [91]. Annexin V is introduced in cell culture medium for a short time. Detached cells are pooled with harvested cells (in case of adherent cells), and propidium iodide (or 7-AAD) is added. Intact, early apoptotic, necrotic, and late apoptotic cells are respectively untagged, fluorescent in green channel, fluorescent in red channel, simultaneously fluorescent in both green and red channels. Quantification of each cell population is performed by flow cytometry. Fluorescent microscopy can give supplementary morphology-based information concerning the cellular viability of epithelial or bronchial cells. Indeed, apoptotic cells are characterized by membrane blebbing.

22.5.3 Genotoxicity

Nanoparticles can penetrate inside cells, causing damage to DNA by single or double strands breaking. The single cell gel assays (SCG) also called "comet assay" are able to detect DNA single strand breaks, alkali labile sites or incomplete excision repair sites with a strong sensitivity to determine the lower DNA damage [92]. However these tests require the extraction of single-strand DNA, obtained after different steps including: (1) cells loading into an agarose gel slice, (2) treatment with a lysis solution, and (3) DNA unwinding with an alkaline buffer [93]. The staining is generally achieved after a neutralization step with fluorescent compounds such as ethidium bromide, propidium iodide, or 4′,6′-diamidino-2-phenylindole (DAPI), or nonfluorescent dyes such as silver nitrate. Quantitative analysis can be performed by determining the proportion of cells with altered migration onto the agarose gel, or by classifying them according to the length of migration. The ability of lung cancer cells to form colonies is another relevant endpoint of cytotoxicity and genotoxicity [94]. A very small amount of treated cells are seeded on plates in order to perform single cell forming colonies. After a long incubation period (few days), cells are fixed to finally count colonies after trypan blue staining [95].

22.5.4 Oxidative Stress

Oxygen is required for all aerobic organisms, but it is also a strong oxidant molecule that can be responsible for undesirable oxidations in cells. This hazardous phenomenon is called oxidative stress [96] and can be caused by free radicals, reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as superoxide anion (O_2^{-}), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorite anion (ClO^{-}), peroxynitrite (NO_3^{-}), and also copper and iron ions (Cu^{2+}, Fe^{3+}) that catalyze Haber–Weiss and Fenton reactions [97] (Fig. 22.8). In physiological conditions, reactive species are neutralized by antioxidants, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), reductase (GPR) and S-transferase (GST), ascorbic acid (vitamin C), retinoids (vitamin A), carotenoids, tocopherols (vitamin E), and selenium. However, when the organism is exposed to hazardous compounds like pollutants, ultraviolet, tobacco smoke, or nanoparticles, the oxidant–antioxidant balance is destabilized, and ROS and RNS are formed.

As it was widely shown in the literature, lungs are the site of several oxidative stress reactions [98–100]. For instance, one of the causes of emphysema or acute respiratory distress syndrome is related to oxidant release in alveolar tissues or pulmonary endothelium [101], and SOD plays an antioxidant key role in asthma and in chronic obstructive pulmonary disease [102].

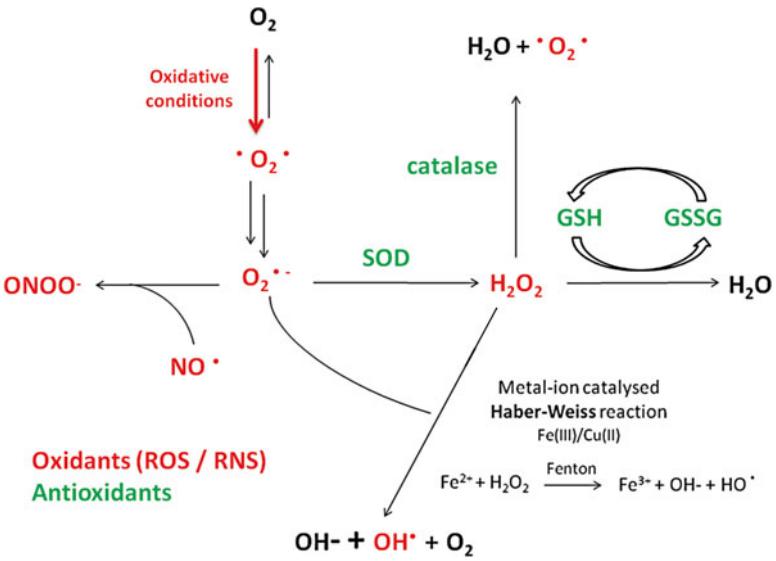

Fig. 22.8 Oxidants (in red) and antioxidants (in green) species involved in oxidative stress reactions. *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *SOD* superoxide dismutase, *GSH/GSSH* reduced sulfhydryl/oxidized disulfide glutathione (modified from [97])

22.5.4.1 Reactive Oxygen Species

To quantify intracellular located ROS, cells are shortly incubated, before exposure to nanoparticles, with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) that penetrates inside cells [103] and which upon cleavage of the acetate group by intracellular esterase is converted into 2′,7′-dichlorofluorescein (DCF) that expresses a high fluorescent intensity after ROS oxidation. Quantification is achieved by flow cytometry.

22.5.4.2 Reactive Nitrogen Species

Contrary to ROS, cells release RNS in the extracellular medium. The most common method to quantify them is the Griess reagent [104, 105] containing sulfanilic acid and α-naphthylamine in acetic acid. In presence of RNS, a pink compound that absorbed at a wavelength of 560 nm will be formed. However, this method has a poor sensitivity, the nitrite concentration being detectable in the range of 1–5 µM. The 2,3-diaminonaphthalene is also able to react with nitrites forming a fluorescent compound (excitation at 355 nm and emission at 460 nm) allowing the detection of lower nitrite concentrations (from 0.02 to 10 µM) [106]. Moreover, this method can quantify the nitrates, by pre-converting them into nitrites with NADPH.

22.5.4.3 Superoxide Dismutase

The SOD catalyzes the dismutation of superoxide radicals ($2O_2^{\cdot-} + 2H + \diamond O_2 + H_2O_2$) [107]. Its increase in the extracellular medium characterizes the presence of superoxide radical that is immediately neutralized by the enzyme. To quantify SOD, superoxide radical anions are formed in situ. They are either neutralized by the SOD, if it is present in the extracellular medium, or they react with nitroblue tetrazolium forming colored nitroblue tetrazolium-formazan (NBT-formazan) (absorbance at a wavelength of 540 nm) [108, 109]. In presence of SOD, production of NBT-formazan is inhibited and the quantity of SOD is accessible by a colorimetric assay.

22.5.4.4 Glutathione

Physiologically, glutathione exists within the cell under, both, the reduced sulfhydryl form (GSH) and the oxidized disulfide form (GSSG). In healthy cells, GSSG is quickly reduced by glutathione reductase into GSH, but in presence of high number of free radicals, GSSG cannot be reduced. GSH can react with the 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the 5-thio-2-nitrobenzoic acid (TNB) (absorbance at a wavelength of 412 nm) and a glutathione derivate (GS-TNB) [110]. The addition of glutathione reductase allows the reduction of GS-TNB into GSH, and the reduction of GSSG into two molecules of GSH. The total quantity of glutathione is [GDH]total = [GSH] + 2[GSSG]. To determine the exact amount of each glutathione form, a second assay can be performed: the excess of 2-vinylpyridine reacting with GSH is neutralized by triethanolamine. GSSG assay is then performed as previously described.

22.5.5 Inflammatory Response

22.5.5.1 Cytokine Production

The immune system is composed of the innate system that is nonspecific and the acquired system that is antibody mediated. After even a slight perturbation in lungs, the immune response is activated and regulated (amplitude and duration) thanks to low molecular weight proteins, called cytokines, released in response to cellular signals [111]. Cytokine denomination includes chemokine, interleukin (IL), growth factor, and interferon (IFN). Every cytokine—that can be secreted in cascade—can cause multiple effects on growth and differentiation (mitosis, chemotaxis, angiogenesis, cytoskeleton arrangement, immunomodulation, and extracellular production) in a large variety of cells, even at low concentration [111, 112]. Pro-inflammatory chemotactic chemokines (IL-8, Monocyte Chemoattractant Protein (MCP)-1) are able to attract primarily neutrophils and cells of the innate immune system, regulate

cell trafficking [113], and can be produced by macrophages or alveolar type-II epithelial-like cells [114, 115]. By regulating the migration and infiltration of monocytes, MCP-1 is also involved in various diseases [116]. The tumor necrosis factor-α (TNF-α), IL-1, IL-6, mostly secreted by alveolar macrophages, are involved in pulmonary diseases; TNF-α increase transitory, with high levels in a short time, but will cause important injuries in lung and fever or tachycardia [111].

The inflammatory response can be quantified by assaying released cytokines in extracellular medium. Cytokines have been historically quantified by ELISA (Enzyme Linked Immunosorbent Assay) based on cytokine recognition with specific antibodies linked to an enzyme detected by a colorimetric reaction. However by ELISA, only one cytokine per sample can be quantified. More recently new methods have been developed to assay several cytokines (until 30) simultaneously [117]. Among them, BD™ CBA (Cytometric Beads Array—BD Biosciences) [118, 119], Luminex® System (Invitrogen) and BioPlex cytokine assays (Bio-Rad) were successfully used. Such systems use calibrated beads of different sizes and fluorescence wavelengths that specifically recognize cytokines. The final addition of antibodies that also recognize each cytokine allows for quantifying the amount of each cytokine. These methods lead to important improvement and a higher sensitivity compared to ELISA tests [117, 120].

22.5.5.2 Complement Activation

The complement system is a multicomponent triggered enzyme cascade that attracts phagocytes to microorganisms increasing capillary permeability, neutrophil chemotaxis and adhesion [112]. As previously shown, epithelial lining fluid contains the complement system proteins [121]. In one example of complement activation determination, bovine heat-inactivated serum exposed to nanoparticles was loaded into the bottom of 96-well neuroprobe chemotaxis chamber, equipped with polycarbonate filter with 5 μm pores. Murine macrophages were placed at the top of the insert, in serum free media. The positive control employed was rich in chemotaxis complement proteins C3a (recognized by phagocytes that will proceed with engulfment of nanoparticles) and C5a (chemotaxis for polymorphonuclear). After incubation, the insert was removed and cells were stained with Romanowsky reagent (a mixture of eosin and methylene blue). Absorbance measurement allows the assessment of the amount of macrophages passed through the filter, which number was function of complement activation.

22.5.5.3 Polymorphonuclear Counting in Bronchoalveolar Lavages

Polymorphonuclear (PMN) are locally recruited in lungs and an increased number characterizes a disorder. By collecting the bronchoalveolar lavages (BAL) on animals, cells can be characterized and counted on a hemocytometer. After nanoparticle lung delivery, BAL are withdrawn from anesthetized animals. Bronchoalveolar lavage fluids (BALF) are then stained with Romanowsky reagent to estimate the number of PMN present.

22.5.6 Mucus Interactions

As the mucus is a strong defense barrier in the pulmonary tract, interactions with inhalable nanoparticles are important, and different parameters can be investigated to characterize mucus alterations. To begin with, an overproduction of mucus is synonym of airway inflammation. Four mucins are generally quantified: MUC2, MUC5AC, MUC5B, and MUC19. The quantification of these proteins or their respective mRNA expression can give essential information about the mucus state. The quantity of mucin in BALF can be obtained by ELISA [122, 123] or electrophoresis [124]. Both methods use multiple anti-mucin antibodies, specific of each mucin proteins. To investigate the passage of insoluble fluorescent particles across the mucus layer, artificial mucus composed of water, DNA, egg yolk emulsion, mucin, pentetic acid (DTPA), NaCl, KCl, and cell culture medium (RPMI) [125] can be prepared and then coated on a gelatin layer [42]. The measurement of the turbidity of the gelatin layer is related to the quantity of particles able to cross the mucus layer. On the other hand, the muco-adhesion of particles can be estimated by mixing them with a known mucin solution [47]. The suspension is then centrifuged, and the supernatant isolated to quantify the free mucin by a Bradford assay. Finally, mucus layer can be labeled with fluorescent wheat germ agglutinin, to be observed in confocal microscopy, after incubation with fluorescent particles [126].

Taken all these methods into account, it is obvious that complete studies are required to deeply understand nanoparticle effects, firstly because all toxicity endpoints are linked, and secondly because eventual interferences of nanoparticles with the assay can lead to false results [127]. All tests have shown their interest to evaluate the toxicity of nonbiodegradable nanoparticles [128] and are subsequently relevant for investigating the safety of biodegradable nanoparticles. For example, titanium dioxide nanoparticles were proved to induce DNA damage [129], oxidative stress [5], inflammatory response [130], and apoptosis [131]. However, these tests have to be taken with caution. For instance artifacts associated to MTT test have been reported [132].

22.6 Models for Lung Nanotoxicology: In Vitro, Ex Vivo, In Vivo

The complexity of the respiratory tract explains the high number of in vitro/in vivo/ex vivo models available to mimic all physiological or histological conditions present in lungs (Table 22.1). Several reviews draw a complete list of in vitro (primary cultured cells; human and animal cell lines isolated from human cancer or transformed from normal cells; cell cocultures), ex vivo (isolated perfused lung—IPL), and in vivo (rodents such as rats, guinea pigs, or mice; larger animals such as rabbits, dogs, monkeys, and sheep) models that can be used to assess pulmonary toxicity [32, 133]. Most of these models were used to study the impact of nonbiodegradable

Table 22.1 In vitro, ex vivo, and in vivo models used for nanotoxicity studies

Models			Organ/origin
In vitro cells			
Epithelial			
	Bronchial	Primary	Isolated from bronchial spaces—human or rodents
		NHBE (*primary cells*)	Isolated from normal human lung
		Beas-2B	Transformed from normal human lung
		16HBE14o-	Transformed from normal human lung
		Calu-3	Human lung adenocarcinoma; derived from metastatic site: pleural effusion
	Alveolar type II	A549	Human lung adenocarcinoma
		RLE-6 TN	Rat lung
		Primary AT I/II	Isolated from alveolar spaces—human or rodents
	Alveolar type II/Clara	NCI H441	Human papillary adenocarcinoma
	Lymph node	H2009	Human lung
Macrophages	Alveolar	Primary	Isolated from human or murine BAL
		NR8383	Rat lung
Ex-vivo			
	Isolated perfused lung		Rat
			Rabbit
			Guinea pig
In vivo			
	Small rodent	Rat	Wistar, Sprague-Dawley
		Mice	Balb/c
		Guinea pig	
	Mammal	Sheep	
		Dogs	Beagle

AT I/II alveolar type I/II epithelial cells, *BAL* bronchoalveolar lavages

nanoparticles—also called manufactured or engineered nanoparticles and particulate matter, and only some of them were applied to study effect of nanoparticles used as drug carriers in lung drug delivery.

22.6.1 In Vitro Cell Culture for Nanoparticle Toxicity Studies

A lot of cell lines used to investigate potential effects after particle exposure are commercially available [134]. The majority of these cell lines are established from cancer tissues and others are immortalized with Epstein-Barr viral coinfection.

In most cases, cell lines are well characterized and present the advantage to be not expensive and easy to use. However, to stay as close as possible to the physiological response, the use of primary cells can be performed as a second approach. Handling these cells is however more complicated than the use of cell lines since they have to be well characterized after sampling and before their use. Moreover, their stability is generally limited to a small number of passages. To mimic the lung, human or murine epithelial cells (bronchial, alveolar, lymph nodes), endothelial cell lines, and murine macrophages are available. Human primary macrophages can be obtained from BAL or by monocyte differentiation.

The delivery to the bronchus can be studied on bronchial epithelial cell lines, such as 16HBE14-o cells, human bronchus cells [74, 135] or Calu-3 cells [136], isolated from a human lung carcinoma [137]. Both these cell lines can be cultured at the air–liquid interface, to form a confluent monolayer with good transepithelial electrical resistance (TEER) values, due to polarized tight junctions [138] and a dense mucus layer [139]. Using these models, good correlations with in vivo results were shown [140]. Gelatin and human serum albumin nanoparticles were prepared and stabilized with glutaraldehyde to deliver drug or gene into primary lung cells (isolated from lobectomies) or 16HBE14o- cells [74]. Previous confocal microscopy observations have evidenced that nanoparticles were taken up according to an endocytic mechanism. Then, toxicity studies on both cell types have shown that membrane integrity was not affected (LDH), even after the highest tested concentration (50 μg/mL), and no IL-8 secretions were detected. Another study has investigated the potential gene delivery polyethylenimine (PEI)-coated PLGA nanoparticles [136]. Nanoparticle uptake was observed within Calu-3 cells with a lysosomal co-localization. The uptake level was shown to be PLGA/PEI ratio-dependent, as demonstrated by the levels of gene expression.

Beas-2 B cells, isolated and modified from normal human bronchial cells [141], were widely used to study the effect of nonbiodegradable nanoparticles such as the inflammatory response after particulate matter exposure [142], the oxidative stress after exposure to silica nanoparticles [143] and complete toxicity studies (oxidative stress, apoptosis, gene expression, DAPI staining) after exposure to titanium dioxide nanoparticles [144]. In most of these studies the model was relevant to demonstrate toxicity properties of the tested particles.

Major studies that focused on alveolar conditions used the A549 cell line. These cells are isolated from a human carcinoma [145] and constitutes a good model of type II pneumocytes [146, 147], giving the possibility to test, after exposure to biodegradable nanoparticles, several previously detailed toxicity endpoints. As an example, the uptake of fluorescent chitosan nanoparticles was shown to be an endocytosis mechanism initiated by adsorptive steps [43], while the SLN (loaded with paclitaxel) endocytosis was PEG or folate coating-mediated [148]. The low cytotoxicity of SLN based on 6-lauroxyhexyl lysinate and complexed to DNA allowed to induce a similar rate of gene transfection comparable with those obtained with Lipofectamine (commercial reagent) [37]. Finally, liposomes loaded with ciprofloxacin have shown a good antibacterial effect without leading to LDH release [149]. Moreover, after exposure of A549 cells to inorganic nanoparticles, apoptosis detection, cell proliferation assay, ROS quantification and inflammatory response were observed [3].

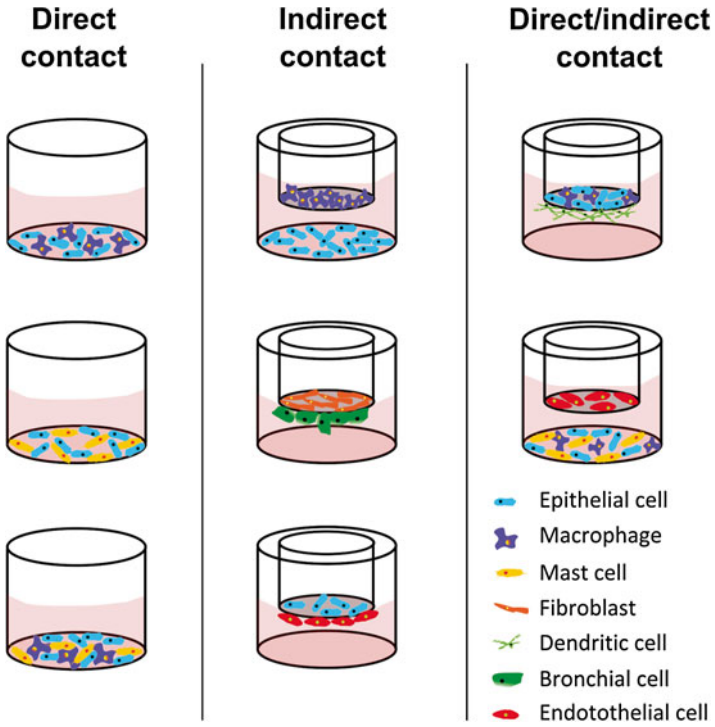

Fig. 22.9 Models used to mimic the pulmonary tract using cell for cell cocultures

To expand alveolar condition studies, human primary alveolar type II cells were isolated after lung resection of carcinoma [150]. Such cells express an alveolar cell-type phenotype and can be immortalized [151] showing an increased internalization of latex beads [152].

22.6.2 Coculture of Lung Cells in Nanotoxicology

Coculture systems have provided significant advances in cell biology. Indeed, by mixing different cell types (two or more), interactions occur and culture conditions are closer to physiology than classic in vitro cell line models. Coculture models can be established with direct or indirect contact between the cells (Fig. 22.9). Models with indirect contact require the use of cell culture inserts (such as Transwell®) equipped with porous membranes allowing communication between different cell types placed in wells. Each cell type is then seeded on each side of the insert. To mimic pulmonary tract, a large variety of cells have been used: bronchial, alveolar, epithelial, fibroblast, dendritic, and macrophages. However, in each case the relevance of cocultures needs to be validated by deep comparisons between cells in monoculture and in coculture.

Bronchial conditions were reproduced by a coculture of 16HBE14o- cells or human primary bronchial cells (apical compartment) with lung fibroblast cells Wi-38 (basolateral compartment) at the air–liquid interface [153]. The use of primary cells has shown interest. Indeed, in coculture with fibroblasts, primary bronchial cells (which are continuously differentiated) mimic the structure of a native polarized bronchial epithelium showing mucus-producing, basal, and ciliated cells. Such model was used from 3 weeks to 3 months [153].

Epiairway™ (MatTek Corporation [154]) and MucilAir™ (Epthelix [155]), two commercial differentiated pulmonary epithelium, are available to perform toxicity tests after exposure to chemical compounds [156] or manufactured nanoparticles [157, 158]. Composed of human primary cells isolated from trachea, bronchus, and nose, MucilAir™ device is cultivated at the air–liquid interface and presents characteristics of airway tissues (available for healthy or damaged conditions). Indeed, the device contains basal, ciliated, and mucosal cells and displays a regular ciliary beating, tight junctions, ion transport, and metabolic activity. The epithelium remains in homeostasis state for more than 1 year, allowing long term studies after single or repeated exposure. After 28 days repeated exposure to chemical compounds, cytochrome expression was modulated [156]. After delivery of nanoparticles on apical surface, transepithelial resistance, LDH and cytokine released were quantified [157]. It was shown that the epithelium has the potential to recover after low nanoparticle concentration exposure. EpiAirway™ and MucilAir™ present an interesting potential to drive environmental studies, as the ones responding to EU regulations in the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) programme [159].

Alveolar conditions were simulated by different coculture models. For example, the indirect coculture of A549 cells or NC H441 with primary human pulmonary microvascular endothelial cells allowed developing a model of an alveolar–capillary barrier [160]. This model has been used to study environmental cadmium exposure side-effects and has shown that whereas viability of epithelial cells largely decreases, the viability of endothelial cells remains unaffected [161]. To investigate the predominant role of resident macrophages following air particulate exposure, lung alveolar epithelial cells, A549 [95, 162], L132 [163], RLE-6N [164], were cocultured with macrophages [differentiated from THP-1 monocytes [162, 165], Mono Mac 6 [162] or human [163]/murine [164] alveolar macrophages isolated from BAL] in direct or indirect contact. In coculture with A549, macrophages differentiated from THP-1 monocytes acquire alveolar macrophage phenotypic characteristics [166], and after exposure to ultrafine nanoparticles, coculture synergistic effects were observed for the release of IL-6 and IL-8 [162], while no modification of ROS and DNA damage levels were noticed [95]. The defensive role of alveolar macrophages against particulate matter was proved in coculture with L132 cells [163]. In coculture, the levels of particulate matter metabolizing enzymes produced were higher than in monoculture. The significant attenuation of IL-1β and IL-6 cytokines secretions after exposure to jet fuel on a coculture of rat alveolar cells and macrophages compared to monoculture of each cells confirmed that interactions between cells exist [164].

Rothen-Rutishauser et al. [167] have established a tri-culture of (1) A549 cells seeded on the apical compartment of Transwell®, (2) differentiated dendritic cells seeded on the basal side of the turned insert, and (3) differentiated macrophages added on the apical surface of the epithelial cell monolayer. This tri-culture was used to investigate effects of nonbiodegradable nanoparticles in alveolar conditions [168]. Following TEM observations, TiO_2 nanoparticles were found in all cell types, whereas carbon nanotubes were present only in epithelial cells. After exposure to carbon nanotubes, ROS production was observed in majority of cells, with a higher level than in monoculture of epithelial cells, whereas IL-8 and TNF-α quantification (ELISA) did not show any synergistic effect.

In order to deeply understand toxicity mechanisms, and more particularly the inflammatory response after exposure to particulate matter, Alfaro-Moreno and coworkers have deeply investigated monoculture and coculture of epithelial cells (A549), macrophages (differentiated THP-1 monocytes), mast cells (HMC-1), and endothelial cells (EAHY926) [169]. Different coculture combinations were performed: mast cells or macrophages with epithelial cells in direct contact; mast and epithelial cells and macrophages in direct contact, with or without endothelial cells in indirect contact. Cytokine release profile was modified according to the combination and the key role of mast cells and macrophages was demonstrated (a large increase of several cytokines in coculture), as well as the activation of endothelial cells by the coculture. The cytokine release profile of the system composed of the four cell types was in accordance with in vivo profile and correlated to asthmatic patients who were exposed to such particulate matter.

22.6.3 Ex Vivo Models

22.6.3.1 Isolated Perfused Lungs

The isolated perfused lung (IPL) system represents an interesting alternative when considering all cell–cell interactions that occurred within the isolated organ [170]. This technique consists in extracting the respiratory system (lungs and/or trachea) from rats, guinea pigs, or rabbits, and keep it as close as possible to the physiological conditions [171]. The pulmonary circulation (air flow rate, temperature, blood circulation) is maintained in a complex track of pumps to provide isolation, perfusion and ventilation of isolated lungs. IPL are not much used in nanotoxicology even though the radioactive tracking of iridium particles to an isolated perfused rat lung has shown a similar particle distribution to the one occurring in vivo, confirming the pertinence of such models [172].

PLGA or branched polyester-based nanoparticles were loaded with a fluorescent compound [173] or salbutamol [174], for the delivery to the isolated perfused rat lung. Kinetic spectroscopic studies on the IPL have confirmed the presence of nanoparticles in deep lungs up to 2 h after administration, and the release of active compounds in two phases, the first corresponding to a constant increasing of

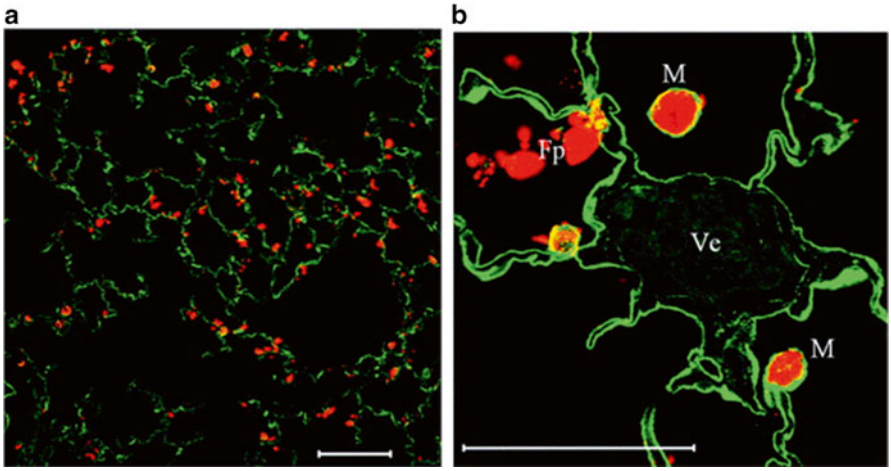

Fig. 22.10 Particle distribution in lungs after intratracheal administration in isolated perfused organs. (**a**) Microspheres in *red* were homogeneously distributed into the lungs. (**b**) Particles were located in macrophages (M) but free particles (Fp) were also observed. No particles were detected in pulmonary vessels (Ve). Bars indicate 50 μm. (Reproduced from [175]).

compounds in the IPL, and the second being the phase of absorption. Similar studies were performed with polystyrene nanoparticles, and confocal laser scanning microscopy imaging of lung slices has shown the presence of such nanoparticles in the deep lung, principally in alveolar macrophages and in free alveolar spaces [175] (Fig. 22.10), but no evidence of translocation from alveolar to the blood circulation was shown [176].

Studies carried out on IPL have certain advantages such as the possibility to achieve complete qualitative and quantitative studies of drug release, by frequent sample collection, but, unfortunately, in a short time duration, because structural and functional integrity of organs will quickly decline [177]. Moreover, physiological conditions are not strictly respected, since lymph flow is altered, bronchial perfusion is suppressed, autonomic innervations are disconnected and blood cells are not present [172]. In addition, the technique of isolation is hard to control and the perfusion system is expensive.

22.6.3.2 Mucus Models

Because of the rigidity of the mucin and the pore size distribution, mucus penetration is different for each nanoparticle. Indeed, interactions are surface coating-mediated [178]. It was for instance shown that PEG coating decreases mucus–nanoparticles interactions [19], while chitosan coating leads to a strong muco-adhesion [47]. Indeed, chitosan-coated nanoparticles displayed more interactions with mucins (confirmed by an increase of zeta potential values), and a more important passage through the mucus than equivalent PVA-coated PLGA nanoparticles [42, 126].

22.6.4 *In Vivo Models*

Compared to other models, in vivo models present the large advantage to be a whole organism. Moreover, as previously described, in vitro lung delivery can be easily performed with specific veterinary devices.

In most cases, small rodents have been employed to follow particle biodistribution and to perform assays on BAL after lung nanoparticle exposure. For example, insulin loaded into liposomes based on hydrogenated soybean phosphatidylcholine and cholesterol [179] or into SLN [180] were administered to diabetic mice or rats by inhalation. In addition of the expected sustained effect, analyses of BAL have shown no immunoreaction nor inflammation (no leukocyte increase). Similarly, liposomes loaded with ciprofloxacin did not induce toxicity after intratracheal delivery to rats [149]. SLN stabilized with PVA and loaded with several antibiotics were also demonstrated to be interesting for the treatment of tuberculosis in guinea pigs [181]. In addition to the sustained effect observed in peripheral organs (until 7 days after delivery), the absence of alteration in serum bilirubin assays has confirmed the safety of the formulations. PVA-coated PLGA nanoparticles loaded with elcatonin have shown low lung retention in guinea pigs whereas chitosan-coated PLGA nanoparticles were more largely retained. The presence of chitosan causes high muco-adhesive properties, may also contribute to open intracellular tight junctions [182] and increase the cellular uptake through strong electrostatic interactions with the negative charge of cellular membranes [81].

Very few studies relate experiments with non-rodents for lung drug delivery of nanoparticles. Beagle dogs were used to follow X-ray contrast agent nanoparticles, stabilized by Pluronic F68 or F108 (poloxamers) in lung draining lymph nodes, and thus detectable with contrast agent radiographs [183]. After administration, the dogs were in good clinical conditions (good appetite, normal breathing, and active/alert behavior) despite the presence of lung lesions detected during the autopsy. These lesions were described as infiltrations of macrophages signing an inflammatory response, more prevalent with Pluronic F108 than F68. Sheep have shown a good lung dynamic compliance after intratracheal administration of liposomes loaded with amikacin [184] and the therapeutic effect of the liposomal form was higher than the free drug without causing any irritation.

22.6.5 *Compared In Vitro/Ex Vivo/In Vivo Nanotoxicity Studies*

Unfortunately, all the models cited previously rapidly reach some limits. Indeed, despite their human origin, in vitro cell models are more often immortalized by genetic modifications or originate from tumoral cancer which means they are quite far from the reality. Coculture models improve traditional in vitro cell culture by mimicking cell–cell interactions. Ex vivo and in vivo models represent an

interesting alternative, thanks to the presence of cell interactions or fluid circulation. Yet the animal physiology is different from the human one. In order to improve our understanding of the behavior and interactions of nanoparticles towards human organism, compiling together the results obtained on different models may represent a strong interest.

For example, Dailey et al. [86] have firstly proved that polymeric nanoparticles (PLGA and branched PLGA) do not induce a significant LDH release after 24 h exposure to A549 cells, whereas in Balb/c mice, 24 h and 14 days after instillation, LDH levels in BAL were slightly higher than for non-treated animals. In addition, nanoparticles did not provoke either PMN recruitment or macrophage inflammatory protein secretion. Nassimi et al. [185] have used the same method to investigate the toxicity of SLN: firstly towards A549 cells, then ex vivo on isolated lung slices and finally on Balb/c mice. In vitro cytotoxicity results have given a higher IC50 than on ex vivo experiments, that was explained by the more basic model, and the cell–cell interactions that definitely play a role in ex vivo models. The three models have shown the absence of LDH release as well as inflammatory response.

The effect of nanoparticle stabilizers (PVA, chitosan, Pluronic F68) coating PLGA nanoparticles was investigated towards bronchial and alveolar cells [77, 126, 186]. Pluronic F68-covered PLGA nanoparticles penetrate more deeply the mucus layer on the top of Calu-3 cells compared to PVA- or chitosan-covered nanoparticles [126], without causing more cytotoxicity, inflammatory response, or mucus glycoproteins mRNA expression [77]. However, the same particles induce an increased inflammatory response on A549 cells with a decrease of the mitochondrial activity, correlated with a higher internalization [186]. Chitosan-coated PLGA nanoparticles are also more cytotoxic than PVA-coated nanoparticles on A549 cells, without inducing other side effects (full membrane integrity, no inflammatory response) [42]. The toxicity of these nanoparticles was explained by the own cytotoxicity of chitosan in aqueous solution. In addition, chitosan-coated nanoparticles caused a reversible decrease of TEER [126]. Additionally, an increase in cell marker membrane and in the IFN-γ production from dendritic cells after delivery of chitosan nanoparticles to rats was reported [46].

In vitro and in vivo comparisons were performed in order to evaluate the radiodiagnostic tool potential of hematoporphyrin linked to albumin under nanoparticle form [44]. Accumulation was shown on A549 cells and correlated to a photodynamic activity after UV exposure, without any toxicity. The accumulation was confirmed in mice-bearing lung tumors and nanoparticles have shown a better half-life in rabbits than the free drug.

As there are in first line to face inhaled nanoparticles, alveolar macrophages should be widely studied, and particularly for phagocytosis (uptake) studies. Rifampicin was encapsulated into PLGA nanoparticles or microparticles [53]. Nanoparticles, or free drug as control, were spray-dried with mannitol to form microparticles. High aerosol performances of rifampicin/PLGA/mannitol microparticles were confirmed with the cascade impactor. In addition, thanks to the immediate mannitol dissolution, rifampicin/PLGA nanoparticles were immediately released. Applied on rat alveolar macrophages (NR8383), PLGA nanoparticles were taken up in significant quantity

but less than PLGA microparticles. When rats were intratracheally administered with the three different formulations, the uptake of rifampicin associated to PLGA/mannitol microparticles was higher than from the other preparations. Microscopy observations of rat lungs evidenced that PLGA microparticles were rapidly excreted by the mucociliary clearance, whereas after mannitol dissolution, PLGA nanoparticles were retained in lungs. In comparison, rifampicin was loaded in SLN of 850 nm and evaluated, in vitro on alveolar macrophages, primary epithelial cells (isolated from rats) and cell lines (NR8383 and A549), and delivered in vivo to rats [187]. As expected, according to the size criteria, alveolar macrophages were specifically targeted (in vitro and in vivo results), with a sustained effect up to 12 h, and without decreasing the mitochondrial activity. It is also important to highlight that no differences were observed on primary cells and cell lines in terms of uptake and cytotoxicity. Moreover, delivery of PVA-coated PLGA and PLA nanoparticles on rats have shown that the uptake is predominantly achieved by alveolar macrophages compared to epithelial cells, without increasing the immune response [188].

22.7 Conclusions

Because of their small size, nanoparticles present a specific potential toxicity towards biological systems. Faced with the need to consolidate all toxicity results obtained after nanoparticle exposure, toxicologists have created a new subdiscipline: nanotoxicology. As shown above, nanoparticle toxicity encompasses several endpoints, and in order to suggest a safe nanomedicine, complete toxicity studies should be performed. For this purpose, to deeply understand interactions between nanomedicine and living organisms, several toxicity endpoints and diverse in vitro, in vivo, and ex vivo models should be compared.

The use of nanoparticles in biopharmaceutical applications is constantly increasing. Nanomedicines, obtained through the use of large variety of drugs and nanoparticles, have a promising future in diagnostic and therapeutic (see Tables 22.2 and 22.3 for summary). Two major types of nanoparticles are currently investigated for lung drug delivery: lipid and polymeric nanoparticles. Liposomes or SLN have the main advantages to be prepared with natural compounds, and loaded with insulin they have been shown as an interesting alternative to iv administration (Table 22.2), without causing important toxicity. As far as polymeric nanoparticles are concerned, the stabilizer plays a key role in the interactions with biological systems (Table 22.2). According to the present report, lipid and polymeric nanoparticles are good candidates as nanocarriers.

Finally, a small number of other nanoparticles (Table 22.3) are proposed for lung delivery. For example, the preparation of contrast agent nanoparticles, stabilized with poloxamers, has revealed a low toxicity. Further experimental and clinical studies are expected to understand human organism–nanoparticle interactions, and thus confirm, or not, the therapeutic and diagnostic potential of such nanomedicine.

Table 22.2 In vitro and in vivo pulmonary toxicity endpoints after exposure to lipid-based (liposomes, SLN) and polymer-based nanoparticles

Composition	Drug	Model (administration mode)	Toxicity endpoints	Références
Liposomes				
HPC Chol	Insulin Amikacin	Normal/diabetic mice (aerosolization) Sheep (nebulization)	No cell morphology modification, no inflammation No toxicity, no irritation, good dynamic compliance and lung resistance	[179, 184]
HSPC DCP	Ciprofloxacin	Rat	No toxicity	[149]
PC DPPC	Rifampicin	A549 cells	No cytotoxicity	[47]
6-laurylhexyl lysiniate	DNA	A549 cells Rat	Low toxicity	[37]
PEG Phosphatidyl-ethanolamine Chol DOPE	Manganese DNA	A549 cells LLC1 cells C57B1/6 mice	Cytoplasmic uptake, no cytotoxicity High lung retention after 48 h	[189]
Solid lipid nanoparticles				
Lipids	Insulin	Rat (diabetic)	No toxicity	[180]
Triglycerides Phospholipids		A549 Balb/c mice	IC 50 (mg/mL) = 3,090 (MTT) – 2,090 (neutral red) – 500 (ex vivo) No increasing of chem. KC, LDH, IL-6, IL-8, TNF-α, total proteins, total cells Toxic effects at 500 μg/animal (no effect until 200 μg)	[190]

(continued)

Table 22.2 (continued)

Composition	Drug	Model (administration mode)	Toxicity endpoints	Références
Lipids	Rifampicin + isoniazid + pyrazinamide	Dunkin Hartley guinea pig	No alteration in serum bilirubin Drug release, with sustained effect, after 45 min	[181]
Lipids	Paclitaxel	A549	PEG and folate enhance cellular uptake without cytotoxicity	[148]
Soy bean lecithin	Rifampicin	N8383 Primary rat AM A549 Primary rat ATII Sprague-Dawley rats	Low mitochondrial activity decreasing Better pharmacologic effects than the free drug Sustained effect until 12 h in AM/N8383 SLN fluorescence intensities higher in macrophages than in AEC Macrophage targeting	[187]
Polymer nanoparticles				
PLGA PVA	Elcatonin Paclitaxel HBsAg	Guinea pig Mice Rat Sprague-Dawley rat	Low lung retention Taken up by alveolar macrophages Basal immune response	[81, 182, 188]
		A549 cells Calu-3 cells HBE cells	Low cytotoxicity and Golgi colocalization	[42, 77, 80, 81, 126, 186, 188]
PLGA CS	Elcatonin Paclitaxel	Guinea pig Mice Rat	Long lung retention May open intracellular tight junctions Strong muco-adhesion	[81, 182]
		A549 cells Calu-3 cells	Uptake increase with CS concentration—energy dependent mechanism Medium/high toxicity	[77, 81, 126, 186]

PLGA PF68		A549 Calu-3	High uptake—energy dependent mechanism Medium/high toxicity, significant inflammatory response, full membrane integrity	[77, 126, 186]
PLA PVA	HBsAg	Sprague-Dawley rat Calu-3	Taken up by alveolar macrophages No immune response	[188]
CS	DNA	Mice—Dendritic cells	Increasing of CD80, CD86, CD83 expression (LPS levels) and INF-γ production	[46]

AM alveolar macrophages, *AT I* alveolar Type II epithelial cells, *Chem.* chemokine, *Chol* cholesterol, *CS* chitosan, *DCP* dicetylphosphate, *DNA* deoxyribonucleic acid, *DNA* deoxyribonucleic acid, *DOPE* 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, *DPPC* 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, *HSPC* hydrogenated soy phosphatidylcholine, *PC* phosphatidylcholine, *PEG* poly(ethyleneglycol), *PF68*, pluronic *F68*, *PLA* poly(lactic acid), *PLGA* poly(lactide-co-glycolique) acid, *PVA* poly(vinylic alcohol)

Table 22.3 In vitro and in vivo pulmonary toxicity endpoints after exposure to other nanoparticles relevant for biomedical applications.

Nanomedicine					
Nanoparticle					
Core	Shell	Drug	Model (administration way)	Nanomedicine toxicity endpoints	References
Gold	Sodium tribasic dihydrate		Mice	Slow clearance Biodistribution: heart > thymus > spleen	[191]
		LPS		Fast clearance Biodistribution: spleen > thymus > heart	
Human serum albumin Porcine gelatin	Glutaraldehyde		16HBE14o- cells Primary bronchial cells	Nanoparticle internalized inside cells (energy dependent mechanism) No cytotoxicity, Full membrane integrity No inflammatory response	[74]
Contrast agent	PF68 PF108	Contrast agent	Beagle dog	Good clinical conditions Lung lesions, inflammation Macrophage infiltrations prevalent with PF 108P than PF68	[183]
Fe_3O_4	PLGA	Quercetin	A549 cells Balb/c mice (nebulization)	No cell morphology modification No cytotoxicity (100 μg/mL) No IL-6 secretion, High GSH levels (transitory)	[192]
Antisense oligonucleotides	Mannose receptor		Rat alveolar macrophages	Full membrane integrity Alveolar macrophages targeting	[193]

LPS lipopolysaccharides, *PF68/PF 108* pluronic F68/F108, *PLGA* poly(lactide-co-glycolide)

References

1. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ (2004) Nanotoxicology. Occup Environ Med 61:727–728
2. Mu L, Sprando R (2010) Application of nanotechnology in cosmetics. Pharm Res 27: 1746–1749
3. Choi SJ, Oh JM, Choy JH (2009) Toxicological effects of inorganic nanoparticles on human lung cancer A549 cells. J Inorg Biochem 103:463–471
4. Lin W, Huang YW, Zhou XD, Ma Y (2006) In vitro toxicity of silica nanoparticles in human lung cancer cells. Toxicol Appl Pharmacol 217:252–259
5. Bhattacharya K, Davoren M, Boertz J, Schins R, Hoffmann E, Dopp E (2009) Titanium dioxide nanoparticles induce oxidative stress and DNA-adduct formation but not DNA-breakage in human lung cells. Part Fibre Toxicol 6:1–11
6. Wang JX, Chen CY, Liu Y, Jiao F, Li W, Lao F, Li YF, Li B, Ge CC, Zhou GQ, Gao YX, Zhao YL, Chai ZF (2008) Potential neurological lesion after nasal instillation of TiO2 nanoparticles in the anatase and rutile crystal phases. Toxicol Lett 183:72–80
7. Bachand GD, Allen A, Bachand M, Achyuthan KE, Seagrave JC, Brozik SM (2012) Cytotoxicity and inflammation in human alveolar epithelial cells following exposure to occupational levels of gold and silver nanoparticles. J Nanopart Res 14
8. Patton JS, Fishburn CS, Weers JG (2004) The lungs as a portal of entry for systemic drug delivery. Proc Am Thorac Soc 1:338–344
9. Patton JS (1996) Mechanisms of macromolecule absorption by the lungs. Adv Drug Deliv Rev 19:3–36
10. Sung JC, Pulliam BL, Edwards DA (2007) Nanoparticles for drug delivery to the lungs. Trends Biotechnol 25:563–570
11. Tsapis N, Bennett D, Jackson B, Weitz DA, Edwards DA (2002) Trojan particles: large porous carriers of nanoparticles for drug delivery. Proc Natl Acad Sci U S A 99:12001–12005
12. Mansour HM, Rhee YS, Wu X (2009) Nanomedicine in pulmonary delivery. Int J Nanomedicine 4:299–319
13. Weibel ER, Gomez DM (1962) Architecture of the human lung: use of quantitative methods establishes fundamental relations between size and number of lung structures. Science 137: 577–585
14. Shelly MP, Lloyd GM, Park GR (1988) A review of the mechanisms and methods of humidification of inspired gases. Intensive Care Med 14:1–9
15. Tsuda T, Noguchi H, Takumi Y, Aochi O (1977) Optimum humidification of air administered to a tracheastomy in dogs: scanning electron microscopy and surfactant studies. Br J Anaesth 49:965–977
16. Patton JS, Byron PR (2007) Inhaling medicines: delivering drugs to the body through the lungs. Nat Rev Drug Discov 6:67–74
17. Rubin BK (2002) Physiology of airway mucus clearance. Respir Care 47:761–768
18. Matsui H, Randell SH, Peretti SW, Davis CW, Boucher RC (1998) Coordinated clearance of periciliary liquid and mucus from airway surfaces. J Clin Invest 102:1125–1131
19. Schuster BS, Suk JS, Woodworth GF, Hanes J (2013) Nanoparticle diffusion in respiratory mucus from humans without lung disease. Biomaterials 34:3439–3446
20. Groneberg DA, Eynott PR, Lim S, Oates T, Wu R, Carlstedt I, Roberts P, Mccann B, Nicholson AG, Harrison BD, Chung KF (2002) Expression of respiratory mucins in fatal status asthmaticus and mild asthma. Histopathology 40:367–373
21. Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER (1982) Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis 126:332–337
22. Sherman MP, Ganz T (1992) Host defense in pulmonary alveoli. Annu Rev Physiol 54: 331–350
23. Wright JR (1997) Immunomodulatory functions of surfactant. Physiol Rev 77:931–962

24. Wright JR (2005) Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 5:58–68
25. Ruge CA, Kirch J, Cañadas O, Schneider M, Perez-Gil J, Schaefer UF, Casals C, Lehr C-M (2011) Uptake of nanoparticles by alveolar macrophages is triggered by surfactant protein A. Nanomedicine 7:690–693
26. Randall TD (2010) Chapter 7 – bronchus-associated lymphoid tissue (BALT): structure and function. In: Sidonia F, Andrea C (eds) Advances in immunology. Academic, New York
27. Samet JM, Cheng PW (1994) The role of airway mucus in pulmonary toxicology. Environ Health Perspect 102(Suppl 2):89–103
28. Sahay G, Alakhova DY, Kabanov AV (2010) Endocytosis of nanomedicines. J Control Release 145:182–195
29. Labiris NR, Dolovich MB (2003) Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications. Br J Clin Pharmacol 56:588–599
30. Yang W, Peters JI, Williams RO 3rd (2008) Inhaled nanoparticles – a current review. Int J Pharm 356:239–247
31. Suarez S, Hickey AJ (2000) Drug properties affecting aerosol behavior. Respir Care 45:652–666
32. Sakagami M (2006) In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. Adv Drug Deliv Rev 58: 1030–1060
33. Byron PR (1986) Prediction of drug residence times in regions of the human respiratory-tract following aerosol inhalation. J Pharm Sci 75:433–438
34. Heyder J, Rudolf G (1984) Mathematical-models of particle deposition in the human respiratory-tract. J Aerosol Sci 15:697–707
35. Heyder J, Gebhart J, Rudolf G, Schiller CF, Stahlhofen W (1986) Deposition of particles in the human respiratory-tract in the size range 0.005–15 μm. J Aerosol Sci 17:811–825
36. Beck-Broichsitter M, Merkel OM, Kissel T (2012) Controlled pulmonary drug and gene delivery using polymeric nano-carriers. J Control Release 161:214–224
37. Li P, Liu DH, Sun XL, Liu CX, Liu YJ, Zhang N (2011) A novel cationic liposome formulation for efficient gene delivery via a pulmonary route. Nanotechnology 22
38. Weers J, Metzheiser B, Taylor G, Warren S, Meers P, Perkins WR (2009) A gamma scintigraphy study to investigate lung deposition and clearance of inhaled amikacin-loaded liposomes in healthy male volunteers. J Aerosol Med Pulm Drug Deliv 22:131–138
39. Bruinenberg P, Serisier D, Cipolla D, Blanchard J (2010) Safety, tolerability and pharmacokinetics of novel liposomal ciprofloxacin formulations for inhalation in healthy volunteers and non-cystic bronchiectasis patients. B49. Bronchiectasis: cystic fibrosis and beyond. Meeting abstracts A3192–A3192
40. Schwarz C, Mehnert W, Lucks JS, Müller RH (1994) Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization. J Control Release 30:83–96
41. Liu J, Gong T, Fu H, Wang C, Wang X, Chen Q, Zhang Q, He Q, Zhang Z (2008) Solid lipid nanoparticles for pulmonary delivery of insulin. Int J Pharm 356:333–344
42. Ungaro F, D'angelo I, Coletta C, D'emmanuele Di Villa Bianca R, Sorrentino R, Perfetto B, Tufano MA, Miro A, La Rotonda MI, Quaglia F (2012) Dry powders based on plga nanoparticles for pulmonary delivery of antibiotics: modulation of encapsulation efficiency, release rate and lung deposition pattern by hydrophilic polymers. J Control Release 157:149–159
43. Huang M, Ma Z, Khor E, Lim L-Y (2002) Uptake of FITC-chitosan nanoparticles by A549 cells. Pharm Res 19:1488–1494
44. Yang SG, Chang JE, Shin B, Park S, Na K, Shim CK (2010) (99)mTc-hematoporphyrin linked albumin nanoparticles for lung cancer targeted photodynamic therapy and imaging. J Mater Chem 20:9042–9046

45. Sahoo SK, Panyam J, Prabha S, Labhasetwar V (2002) Residual polyvinyl alcohol associated with poly (d, l-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release 82:105–114
46. Bivas-Benita M, Van Meijgaarden KE, Franken KL, Junginger HE, Borchard G, Ottenhoff TH, Geluk A (2004) Pulmonary delivery of chitosan-DNA nanoparticles enhances the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of Mycobacterium tuberculosis. Vaccine 22:1609–1615
47. Zaru M, Mourtas S, Klepetsanis P, Fadda AM, Antimisiaris SG (2007) Liposomes for drug delivery to the lungs by nebulization. Eur J Pharm Biopharm 67:655–666
48. Schwab JA, Zenkel M (1998) Filtration of particulates in the human nose. Laryngoscope 108:120–124
49. Nowacki C, Heights A, Brisson AG (1985) Meterd dose inhaler. USA patent application 574,340
50. Sharma K, Somavarapu S, Colombani A, Govind N, Taylor KM (2012) Crosslinked chitosan nanoparticle formulations for delivery from pressurized metered dose inhalers. Eur J Pharm Biopharm 81:74–81
51. Bivas-Benita M, Zwier R, Junginger HE, Borchard G (2005) Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. Eur J Pharm Biopharm 61:214–218
52. Newhouse MT (1992) Powder inhaler. USA patent application 484,069
53. Ohashi K, Kabasawa T, Ozeki T, Okada H (2009) One-step preparation of rifampicin/poly(lactic-co-glycolic acid) nanoparticle-containing mannitol microspheres using a four-fluid nozzle spray drier for inhalation therapy of tuberculosis. J Control Release 135:19–24
54. Anton N, Jakhmola A, Vandamme TF (2012) Trojan microparticles for drug delivery. Pharmaceutics 4:1–25
55. Grenha A, Seijo B, Remuñán-López C (2005) Microencapsulated chitosan nanoparticles for lung protein delivery. Eur J Pharm Sci 25:427–437
56. Tomoda K, Ohkoshi T, Nakajima T, Makino K (2008) Preparation and properties of inhalable nanocomposite particles: effects of the size, weight ratio of the primary nanoparticles in nanocomposite particles and temperature at a spray-dryer inlet upon properties of nanocomposite particles. Colloids Surf B Biointerfaces 64:70–76
57. Tomoda K, Ohkoshi T, Hirota K, Sonavane GS, Nakajima T, Terada H, Komuro M, Kitazato K, Makino K (2009) Preparation and properties of inhalable nanocomposite particles for treatment of lung cancer. Colloids Surf B Biointerfaces 71:177–182
58. Gómez-Gaete C, Fattal E, Silva L, Besnard M, Tsapis N (2008) Dexamethasone acetate encapsulation into Trojan particles. J Control Release 128:41–49
59. Zeng X-M, Macritchie HB, Marriott C, Martin GP (2007) Humidity-induced changes of the aerodynamic properties of dry powder aerosol formulations containing different carriers. Int J Pharm 333:45–55
60. Shi L, Plumley CJ, Berkland C (2007) Biodegradable nanoparticle flocculates for dry powder aerosol formulation. Langmuir 23:10897–10901
61. Ely L, Roa W, Finlay WH, Löbenberg R (2007) Effervescent dry powder for respiratory drug delivery. Eur J Pharm Biopharm 65:346–353
62. Al-Hallak MHDK, Sarfraz MK, Azarmi S, Roa WH, Finlay WH, Rouleau C, Löbenberg R (2012) Distribution of effervescent inhalable nanoparticles after pulmonary delivery: an in vivo study. Ther Deliv 3:725–734
63. Kim I, Byeon HJ, Kim TH, Lee ES, Oh KT, Shin BS, Lee KC, Youn YS (2012) Doxorubicin-loaded highly porous large PLGA microparticles as a sustained-release inhalation system for the treatment of metastatic lung cancer. Biomaterials 33:5574–5583
64. Yang Y, Bajaj N, Xu P, Ohn K, Tsifansky MD, Yeo Y (2009) Development of highly porous large PLGA microparticles for pulmonary drug delivery. Biomaterials 30:1947–1953
65. Hein S, Bur M, Schaefer UF, Lehr C-M (2011) A new pharmaceutical aerosol deposition device on cell cultures (PADDOCC) to evaluate pulmonary drug absorption for metered dose dry powder formulations. Eur J Pharm Biopharm 77:132–138

66. Cooney DJ, Hickey AJ (2011) Cellular response to the deposition of diesel exhaust particle aerosols onto human lung cells grown at the air–liquid interface by inertial impaction. Toxicol In Vitro 25:1953–1965
67. Ungaro F, Giovino C, Coletta C, Sorrentino R, Miro A, Quaglia F (2010) Engineering gas-foamed large porous particles for efficient local delivery of macromolecules to the lung. Eur J Pharm Sci 41:60–70
68. Tseng C-L, Wu SY-H, Wang W-H, Peng C-L, Lin F-H, Lin C-C, Young T-H, Shieh M-J (2008) Targeting efficiency and biodistribution of biotinylated-EGF-conjugated gelatin nanoparticles administered via aerosol delivery in nude mice with lung cancer. Biomaterials 29:3014–3022
69. Varshosaz J, Ghaffari S, Mirshojaei SF, Jafarian A, Atyabi F, Kobarfard F, Azarmi S (2013) Biodistribution of amikacin solid lipid nanoparticles after pulmonary delivery. BioMed Res Int 2013:8
70. Muhlfeld C, Gehr P, Rothen-Rutishauser B (2008) Translocation and cellular entering mechanisms of nanoparticles in the respiratory tract. Swiss Med Wkly 138:387–391
71. Kunzli N, Tager IB (2005) Air pollution: from lung to heart. Swiss Med Wkly 135:697–702
72. Choi HS, Ashitate Y, Lee JH, Kim SH, Matsui A, Insin N, Bawendi MG, Semmler-Behnke M, Frangioni JV, Tsuda A (2010) Rapid translocation of nanoparticles from the lung airspaces to the body. Nat Biotechnol 28:1300–1303
73. Hillaireau H, Couvreur P (2009) Nanocarriers' entry into the cell: relevance to drug delivery. Cell Mol Life Sci 66:2873–2896
74. Brzoska M, Langer K, Coester C, Loitsch S, Wagner TOF, Mallinckrodt CV (2004) Incorporation of biodegradable nanoparticles into human airway epithelium cells—in vitro study of the suitability as a vehicle for drug or gene delivery in pulmonary diseases. Biochem Biophys Res Commun 318:562–570
75. Iversen T-G, Skotland T, Sandvig K (2011) Endocytosis and intracellular transport of nanoparticles: present knowledge and need for future studies. Nano Today 6:176–185
76. Panyam J, Sahoo SK, Prabha S, Bargar T, Labhasetwar V (2003) Fluorescence and electron microscopy probes for cellular and tissue uptake of poly(d, l-lactide-co-glycolide) nanoparticles. Int J Pharm 262:1–11
77. Mura S, Hillaireau H, Nicolas J, Le Droumaguet B, Gueutin C, Zanna S, Tsapis N, Fattal E (2011) Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. Int J Nanomedicine 6:2591–2605
78. Reul R, Tsapis N, Hillaireau H, Sancey L, Mura S, Recher M, Nicolas J, Coll J-L, Fattal E (2012) Near infrared labeling of PLGA for in vivo imaging of nanoparticles. Polym Chem 3:694–702
79. Cell Staining Simulation Tool Life Technologies (2013) Cell staining simulation tool [Online]. http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Cell-Staining-Tool.html?CID=fl-cellstaintool. Accessed 11 Jul 2013
80. CARTIERA MS, Johnson KM, Rajendran V, Caplan MJ, Saltzman WM (2009) The uptake and intracellular fate of PLGA nanoparticles in epithelial cells. Biomaterials 30:2790–2798
81. Yang R, Yang S-G, Shim W-S, Cui F, Cheng G, Kim I-W, Kim D-D, Chung S-J, Shim C-K (2009) Lung-specific delivery of paclitaxel by chitosan-modified PLGA nanoparticles via transient formation of microaggregates. J Pharm Sci 98:970–984
82. Semmler-Behnke M, Takenaka S, Fertsch S, Wenk A, Seitz J, Mayer P, Oberdorster G, Kreyling WG (2007) Efficient elimination of inhaled nanoparticles from the alveolar region: evidence for interstitial uptake and subsequent reentrainment onto airways epithelium. Environ Health Perspect 115:728–733
83. Semmler M, Seitz J, Erbe F, Mayer P, Heyder J, Oberdorster G, Kreyling WG (2004) Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs. Inhal Toxicol 16:453–459
84. Tennant JR (1964) Evaluation of the trypan blue technique for determination of cell viability. Transplantation 2:685–694

85. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
86. Dailey LA, Jekel N, Fink L, Gessler T, Schmehl T, Wittmar M, Kissel T, Seeger W (2006) Investigation of the proinflammatory potential of biodegradable nanoparticle drug delivery systems in the lung. Toxicol Appl Pharmacol 215:100–108
87. Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
88. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 148:2207–2216
89. Jänicke RU, Sprengart ML, Wati MR, Porter AG (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273: 9357–9360
90. Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, Vanoers MHJ (1994) Annexin-V for flow cytometric detection of phosphatidylserine expression on B-cells undergoing apoptosis. Blood 84:1415–1420
91. Vanengeland M, Ramaekers FCS, Schutte B, Reutelingsperger CPM (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 24:131–139
92. Singh NP, Mccoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191
93. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221
94. Shoemaker RH, Wolpert-Defilippes MK, Kern DH, Lieber MM, Makuch RW, Melnick NR, Miller WT, Salmon SE, Simon RM, Venditti JM, von Hoff DD (1985) Application of a human tumor colony-forming assay to new drug screening. Cancer Res 45:2145–2153
95. Jantzen K, Roursgaard M, Desler C, Loft S, Rasmussen LJ, Møller P (2012) Oxidative damage to DNA by diesel exhaust particle exposure in co-cultures of human lung epithelial cells and macrophages. Mutagenesis 27:693–701
96. Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp Physiol 82:291–295
97. Sorg O (2004) Oxidative stress: a theoretical model or a biological reality? C R Biol 327:649–662
98. Li N, Xia T, Nel AE (2008) The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 44:1689–1699
99. Macnee W (2001) Oxidative stress and lung inflammation in airways disease. Eur J Pharmacol 429:195–207
100. Repine JE, Bast A, Lankhorst IDA (1997) Oxidative stress in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 156:341–357
101. Babior BM (2000) Phagocytes and oxidative stress. Am J Med 109:33–44
102. Tsukagoshi H, Shimizu Y, Iwamae S, Hisada T, Ishizuka T, Iizuka K, Dobashi K, Mori M (2000) Evidence of oxidative stress in asthma and COPD: potential inhibitory effect of theophylline. Respir Med 94:584–588
103. Lebel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5:227–231
104. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 126: 131–138
105. Hensley K, Mou S, Pye Q (2003) Nitrite determination by colorimetric and fluorometric griess diazotization assays. In: Hensley K, Floyd R (eds) Methods in biological oxidative stress. Humana, New York

106. Nussler AK, Glanemann M, Schirmeier A, Liu L, Nussler NC (2006) Fluorometric measurement of nitrite/nitrate by 2,3-diaminonaphthalene. Nat Protoc 1:2223–2226
107. Mccord JM, Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049–6055
108. Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287
109. Sun Y, Oberley LW, Li Y (1988) A simple method for clinical assay of superoxide dismutase. Clin Chem 34:497–500
110. Rahman I, Kode A, Biswas SK (2007) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat Protoc 1:3159–3165
111. Kelley J (1990) Cytokines of the lung. Am Rev Respir Dis 141:765–788
112. Galley HF, Webster NR (1996) The immuno-inflammatory cascade. Br J Anaesth 77:11–16
113. Deshmane SL, Kremlev S, Amini S, Sawaya BE (2009) Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 29:313–326
114. Cromwell O, Hamid Q, Corrigan CJ, Barkans J, Meng Q, Collins PD, Kay AB (1992) Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. Immunology 77:330–337
115. Sallusto F, Mackay CR, Lanzavecchia A (2000) The role of chemokine receptors in primary, effector, and memory immune responses. Annu Rev Immunol 18:593–620
116. Polito AJ, Proud D (1998) Epithelial cells as regulators of airway inflammation. J Allergy Clin Immunol 102:714–718
117. Dupont NC, Wang K, Wadhwa PD, Culhane JF, Nelson EL (2005) Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: determinations of a panel of nine cytokines in clinical sample culture supernatants. J Reprod Immunol 66:175–191
118. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, Wilson J, Lowe L, Chen R, Shivraj L, Agadir A, Campos R, Ernst D, Gaur A (2004) Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol 110:252–266
119. Tarnok A, Hambsch J, Chen R, Varro R (2003) Cytometric bead array to measure six cytokines in twenty-five microliters of serum. Clin Chem 49:1000–1002
120. Djoba Siawaya JF, Roberts T, Babb C, Black G, Golakai HJ, Stanley K, Bapela NB, Hoal E, Parida S, Van Helden P, Walzl G (2008) An evaluation of commercial fluorescent bead-based luminex cytokine assays. PLoS One 3:e2535
121. Robertson J, Caldwell JR, Castle JR, Waldman RH (1976) Evidence for the presence of components of the alternative (Properdin) pathway of complement activation in respiratory secretions. J Immunol 117:900–903
122. Lin H, Carlson DM, St George JA, Plopper CG, Wu R (1989) An ELISA method for the quantitation of tracheal mucins from human and nonhuman primates. Am J Respir Cell Mol Biol 1:41–48
123. Phillips JE, Case NR, Celly C, Chapman RW, Hey JA, Minnicozzi M (2006) An enzyme-linked immunosorbent assay (ELISA) for the determination of mucin levels in bronchoalveolar lavage fluid. J Pharmacol Toxicol Methods 53:160–167
124. Spurr-Michaud S, Argüeso P, Gipson I (2007) Assay of mucins in human tear fluid. Exp Eye Res 84:939–950
125. Yang Y, Tsifansky MD, Shin S, Lin Q, Yeo Y (2011) Mannitol-guided delivery of ciprofloxacin in artificial cystic fibrosis mucus model. Biotechnol Bioeng 108:1441–1449
126. Mura S, Hillaireau H, Nicolas J, Kerdine-Romer S, Le Droumaguet B, Delomenie C, Nicolas V, Pallardy M, Tsapis N, Fattal E (2011) Biodegradable nanoparticles meet the bronchial airway barrier: how surface properties affect their interaction with mucus and epithelial cells. Biomacromolecules 12:4136–4143
127. Wörle-Knirsch JM, Pulskamp K, Krug HF (2006) Oops they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Lett 6:1261–1268
128. Hoet PH, Bruske-Hohlfeld I, Salata OV (2004) Nanoparticles - known and unknown health risks. J Nanobiotechnol 2:12

129. Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH (2009) Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res 69: 8784–8789
130. Park E-J, Yoon J, Choi K, Yi J, Park K (2009) Induction of chronic inflammation in mice treated with titanium dioxide nanoparticles by intratracheal instillation. Toxicology 260: 37–46
131. Shi Y, Wang F, He J, Yadav S, Wang H (2010) Titanium dioxide nanoparticles cause apoptosis in BEAS-2B cells through the caspase 8/t-Bid-independent mitochondrial pathway. Toxicol Lett 196:21–27
132. Simon-Deckers A, Gouget B, Mayne-L'HERMITE M, Herlin-Boime N, Reynaud C, Carriere M (2008) In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes. Toxicology 253:137–146
133. Fisher GL, Placke ME (1987) In vitro models of lung toxicity. Toxicology 47:71–93
134. Phelps RM, Johnson BE, Ihde DC, Gazdar AF, Carbone DP, Mcclintock PR, Linnoila RI, Matthews MJ, Bunn PA, Carney D, Minna JD, Mulshine JL (1996) Nci-navy medical oncology branch cell line data base. J Cell Biochem 63:32–91
135. Dombu C, Carpentier R, Betbeder D (2012) Influence of surface charge and inner composition of nanoparticles on intracellular delivery of proteins in airway epithelial cells. Biomaterials 33:9117–9126
136. Bivas-Benita M, Romeijn S, Junginger HE, Borchard G (2004) PLGA-PEI nanoparticles for gene delivery to pulmonary epithelium. Eur J Pharm Biopharm 58:1–6
137. Fogh J, Fogh JM, Orfeo T (1977) One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J Natl Cancer Inst 59:221–226
138. Foster KA, Avery ML, Yazdanian M, Audus KL (2000) Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. Int J Pharm 208:1–11
139. Fiegel J, Ehrhardt C, Schaefer U, Lehr C-M, Hanes J (2003) Large porous particle impingement on lung epithelial cell monolayers—toward improved particle characterization in the lung. Pharm Res 20:788–796
140. Mathias NR, Timoszyk J, Stetsko PI, Megill JR, Smith RL, Wall DA (2002) Permeability characteristics of Calu-3 human bronchial epithelial cells: in vitro - in vivo correlation to predict lung absorption in rats. J Drug Target 10:31–40
141. Reddel RR, Ke Y, Gerwin BI, Mcmenamin MG, Lechner JF, Su RT, Brash DE, Park JB, Rhim JS, Harris CC (1988) Transformation of human bronchial epithelial cells by infection with sv40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res 48:1904–1909
142. Steerenberg PA, Zonnenberg JA, Dormans JA, Joon PN, Wouters IM, Van Bree L, Scheepers PT, Van Loveren H (1998) Diesel exhaust particles induced release of interleukin 6 and 8 by (primed) human bronchial epithelial cells (BEAS 2B) in vitro. Exp Lung Res 24:85–100
143. Eom H-J, Choi J (2009) Oxidative stress of silica nanoparticles in human bronchial epithelial cell, Beas-2B. Toxicol In Vitro 23:1326–1332
144. Park E-J, Yi J, Chung K-H, Ryu D-Y, Choi J, Park K (2008) Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicol Lett 180: 222–229
145. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G (1976) A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 17:62–70
146. Foster KA, Oster CG, Mayer MM, Avery ML, Audus KL (1998) Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. Exp Cell Res 243:359–366
147. Rothen-Rutishauser B, Clift MJD, Jud C, Fink A, Wick P (2012) Human epithelial cells in vitro – are they an advantageous tool to help understand the nanomaterial-biological barrier interaction? Eur Nanotoxicol Lett 4:1–20
148. Yuan H, Miao J, Du YZ, You J, Hu FQ, Zeng S (2008) Cellular uptake of solid lipid nanoparticles and cytotoxicity of encapsulated paclitaxel in A549 cancer cells. Int J Pharm 348:137–145

149. Chono S, Tanino T, Seki T, Morimoto K (2006) Influence of particle size on drug delivery to rat alveolar macrophages following pulmonary administration of ciprofloxacin incorporated into liposomes. J Drug Target 14:557–566
150. Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD (2004) Primary human alveolar type II epithelial cell chemokine release. Am J Respir Cell Mol Biol 30:500–509
151. O'hare MJ, Bond J, Clarke C, Takeuchi Y, Atherton AJ, Berry C, Moody J, Silver ARJ, Davies DC, Alsop AE, Neville AM, Jat PS (2001) Conditional immortalization of freshly isolated human mammary fibroblasts and endothelial cells. Proc Natl Acad Sci 98:646–651
152. Kemp SJ, Thorley AJ, Gorelik J, Seckl MJ, O'HARE MJ, Arcaro A, Korchev Y, Goldstraw P, Tetley TD (2008) Immortalization of human alveolar epithelial cells to investigate nanoparticle uptake. Am J Respir Cell Mol Biol 39:591–597
153. Pohl C, Hermanns MI, Uboldi C, Bock M, Fuchs S, Dei-Anang J, Mayer E, Kehe K, Kummer W, Kirkpatrick CJ (2009) Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit. Eur J Pharm Biopharm 72:339–349
154. Mattek_Corporation. EpiAirway™ [Online]. http://www.mattek.com/456-an-in-vitro-model-of-human-airway-epithelium-epiairway-for-in-vitro-metabolism-toxicity-screening-and-drug-delivery-applications. Accessed 29 Sept 2013
155. Epthelix. MucilAir™ [Online]. http://www.epithelix.com/content/view/6/5/lang,en/. Accessed 29 Sept 2013
156. Constant S, Huang S, Caulfuty M, Bonfante R, Monachino M, Frauenfelder R, Wiszniewski L (2011) 28 Day repeated dose toxicity test on an in vitro cell model. Toxicol Lett 205 (Suppl):S58
157. Constant S, Huang S, Caulfuty M, Bonfante R, Monachino M, Frauenfelder R, Wiszniewski L (2011) A simple method for testing the toxicity of nanomaterials on 3D air–liquid interface human airway epithelia (MucilAir™). Toxicol Lett 205(Suppl):S284
158. Hayden P, Kaluzhny Y, Kubilus J, Ayehunie S, Kandarova H, Klausner M (2011) Use of normal human 3-dimensional (NHu-3D) tissue models (EpiDerm, EpiAirway) for nanotoxicology applications. Alternative to experimental animals. MatTek Corporation, Montréal, QC
159. Bérubé K, Prytherch Z, Job C, Hughes T (2010) Human primary bronchial lung cell constructs: the new respiratory models. Toxicology 278:311–318
160. Hermanns MI, Unger RE, Kehe K, Peters K, Kirkpatrick CJ (2004) Lung epithelial cell lines in coculture with human pulmonary microvascular endothelial cells: development of an alveolo-capillary barrier in vitro. Lab Invest 84:736–752
161. Papritz M, Pohl C, Wübbeke C, Moisch M, Hofmann H, Hermanns MI, Thiermann H, Kirkpatrick CJ, Kehe K (2010) Side-specific effects by cadmium exposure: apical and basolateral treatment in a coculture model of the blood–air barrier. Toxicol Appl Pharmacol 245:361–369
162. Wottrich R, Diabaté S, Krug HF (2004) Biological effects of ultrafine model particles in human macrophages and epithelial cells in mono- and co-culture. Int J Hyg Environ Health 207:353–361
163. Abbas I, Saint-Georges F, Billet S, Verdin A, Mulliez P, Shirali P, Garçon G (2009) Air pollution particulate matter (PM2.5)-induced gene expression of volatile organic compound and/or polycyclic aromatic hydrocarbon-metabolizing enzymes in an in vitro coculture lung model. Toxicol In Vitro 23:37–46
164. Wang S, Young RS, Sun NN, Witten ML (2002) In vitro cytokine release from rat type II pneumocytes and alveolar macrophages following exposure to JP-8 jet fuel in co-culture. Toxicology 173:211–219
165. Stříž I, Slavčev A, Kalanin J, Jarešová M, Rennard SI (2001) Cell–cell contacts with epithelial cells modulate the phenotype of human macrophages. Inflammation 25:241–246
166. Striz I, Wang YM, Teschler H, Sorg C, Costabel U (1993) Phenotypic markers of alveolar macrophage maturation in pulmonary sarcoidosis. Lung 171:293–303
167. Rothen-Rutishauser BM, Kiama SG, Gehr P (2005) A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol 32:281–289

168. Müller L, Riediker M, Wick P, Mohr M, Gehr P, Rothen-Rutishauser B (2010) Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways. J R Soc Interface 7:S27–S40
169. Alfaro-Moreno E, Nawrot TS, Vanaudenaerde BM, Hoylaerts MF, Vanoirbeek JA, Nemery B, Hoet PHM (2008) Co-cultures of multiple cell types mimic pulmonary cell communication in response to urban pm10. Eur Respir J 32:1184–1194
170. Mehendale HM, Angevine LS, Ohmiya Y (1981) The isolated perfused lung—a critical evaluation. Toxicology 21:1–36
171. Byron PR, Roberts NS, Clark AR (1986) An isolated perfused rat lung preparation for the study of aerosolized drug deposition and absorption. J Pharm Sci 75:168–171
172. Meiring JJ, Borm PJ, Bagate K, Semmler M, Seitz J, Takenaka S, Kreyling WG (2005) The influence of hydrogen peroxide and histamine on lung permeability and translocation of iridium nanoparticles in the isolated perfused rat lung. Part Fibre Toxicol 2:3
173. Beck-Broichsitter M, Gauss J, Packhaeuser CB, Lahnstein K, Schmehl T, Seeger W, Kissel T, Gessler T (2009) Pulmonary drug delivery with aerosolizable nanoparticles in an ex vivo lung model. Int J Pharm 367:169–178
174. Beck-Broichsitter M, Gauss J, Gessler T, Seeger W, Kissel T, Schmehl T (2010) Pulmonary targeting with biodegradable salbutamol-loaded nanoparticles. J Aerosol Med Pulm Drug Deliv 23:47–57
175. Hamoir J, Nemmar A, Halloy D, Wirth D, Vincke G, Vanderplasschen A, Nemery B, Gustin P (2003) Effect of polystyrene particles on lung microvascular permeability in isolated perfused rabbit lungs: role of size and surface properties. Toxicol Appl Pharmacol 190: 278–285
176. Nemmar A, Hamoir J, Nemery B, Gustin P (2005) Evaluation of particle translocation across the alveolo-capillary barrier in isolated perfused rabbit lung model. Toxicology 208: 105–113
177. Beck-Broichsitter M, Schmehl T, Seeger W, Gessler T (2011) Evaluating the controlled release properties of inhaled nanoparticles using isolated, perfused, and ventilated lung models. J Nanomater 2011
178. Kirch J, Schneider A, Abou B, Hopf A, Schaefer UF, Schneider M, Schall C, Wagner C, Lehr C-M (2012) Optical tweezers reveal relationship between microstructure and nanoparticle penetration of pulmonary mucus. Proc Natl Acad Sci 109:18355–18360
179. Huang YY, Wang CH (2006) Pulmonary delivery of insulin by liposomal carriers. J Control Release 113:9–14
180. Bi R, Shao W, Wang Q, Zhang N (2009) Solid lipid nanoparticles as insulin inhalation carriers for enhanced pulmonary delivery. J Biomed Nanotechnol 5:84–92
181. Pandey R, Khuller GK (2005) Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis. Tuberculosis (Edinb) 85:227–234
182. Yamamoto H, Kuno Y, Sugimoto S, Takeuchi H, Kawashima Y (2005) Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. J Control Release 102:373–381
183. Mcintire GL, Bacon ER, Toner JL, Cornacoff JB, Losco PE, Illig KJ, Nikula KJ, Muggenburg BA, Ketai L (1998) Pulmonary delivery of nanoparticles of insoluble, iodinated CT x-ray contrast agents to lung draining lymph nodes in dogs. J Pharm Sci 87:1466–1470
184. Schreier H, Mcnicol KJ, Ausborn M, Soucy DM, Derendorf H, Stecenko AA, Gonzalezrothi RJ (1992) Pulmonary delivery of amikacin liposomes and acute liposome toxicity in the sheep. Int J Pharm 87:183–193
185. Nassimi M, Schleh C, Lauenstein H-D, Hussein R, Lübbers K, Pohlmann G, Switalla S, Sewald K, Müller M, Krug N, Müller-Goymann CC, Braun A (2009) Low cytotoxicity of solid lipid nanoparticles in in vitro and ex vivo lung models. Inhal Toxicol 21:104–109
186. Grabowski N, Hillaireau H, Vergnaud J, Aragao LS, Kerdine-Romer S, Pallardy M, Tsapis N, Fattal E (2013) Toxicity of surface-modified PLGA nanoparticles toward lung alveolar epithelial cells. Int J Pharm 454(2):686–694

187. Chuan J, Li Y, Yang L, Sun X, Zhang Q, Gong T, Zhang Z (2013) Enhanced rifampicin delivery to alveolar macrophages by solid lipid nanoparticles. J Nanopart Res 15:1–9
188. Thomas C, Rawat A, Hope-Weeks L, Ahsan F (2010) Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine. Mol Pharm 8:405–415
189. Howell M, Mallela J, Wang C, Ravi S, Dixit S, Garapati U, Mohapatra S (2013) Manganese-loaded lipid-micellar theranostics for simultaneous drug and gene delivery to lungs. J Control Release 167:210–218
190. Nassimi M, Schleh C, Lauenstein HD, Hussein R, Hoymann HG, Koch W, Pohlmann G, Krug N, Sewald K, Rittinghausen S, Braun A, Muller-Goymann C (2010) A toxicological evaluation of inhaled solid lipid nanoparticles used as a potential drug delivery system for the lung. Eur J Pharm Biopharm 75:107–116
191. Hussain S, Vanoirbeek JAJ, Haenen S, Haufroid V, Boland S, Marano F, Nemery B, Hoet PHM (2013) Prior lung inflammation impacts on body distribution of gold nanoparticles. BioMed Res Int 2013:6
192. Verma NK, Crosbie-Staunton K, Satti A, Gallagher S, Ryan KB, Doody T, Mcatamney C, Macloughlin R, Galvin P, Burke CS, Volkov Y, Gun'ko YK (2013) Magnetic core-shell nanoparticles for drug delivery by nebulization. J Nanobiotechnol 11:1
193. Liang W, Shi X, Deshpande D, Malanga CJ, Rojanasakul Y (1996) Oligonucleotide targeting to alveolar macrophages by mannose receptor-mediated endocytosis. Biochim Biophys Acta Biomembr 1279:227–234

Chapter 23
Nanotoxicology: Contemporary Issues and Future Directions

Chandraiah Godugu, Raman Preet Singh, and Ramarao Poduri

Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
Akt	Protein kinase B
AP-1	Activator protein 1
BAL	Bronchoalveolar lavage
BaP	Benzo[a]pyrene
BBB	Blood brain barrier
CNTs	Carbon nanotubes
F-CNTs	Functionalized CNTs
GRAS	Generally regarded as safe
IL	Interleukins
LDL	Low density lipoproteins
MAPK	Mitogen activated protein kinase
MCP 1	Monocyte chemoattractant protein-1
MMP	Mitochondrial membrane potential
MN	Micronucleus

C. Godugu (✉)
National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, Andhra Pradesh, India
e-mail: chandragodugu@gmail.com

R.P. Singh
Evaluserve SEZ (Gurgaon) Pvt. Ltd., Sector - 48, Gurgaon, India

R. Poduri
Centre for Chemical and Pharmaceutical Sciences, Central University of Punjab, Mansa Road, Bathinda 151 001, Punjab, India
e-mail: ramaraop@yahoo.com

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*, Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5_23

mt DNA	Mitochondrial DNA
MWCNTs	Multi walled carbon nanotubes
NF-κB	Nuclear factor-κB
NIR	Near infrared
NPs	Nanoparticles
PARP	Poly ADP ribose polymerase
PDGF	Platelet derived growth factor
PLGA	Poly(lactic-co-glycolic acid)
QDs	Quantum dots
RES	Reticuloendothelial system
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SWCNTs	Single walled carbon nanotubes
TEM	Transmission electron microscope
TNF-α	Tumor necrosis factor alpha
α-SMA	Alpha smooth muscle actin

23.1 Introduction

Nanoparticles (NPs) have existed in nature since antiquity: in volcanic ash [1], particulate matter suspended in air [2], stellar dust etc [3]. In recent years, there has been enormous excitement about the applications of nanotechnology in various disciplines of science, with plethora of speculated applications. The applications of nanotechnology are profound, ranging from information technology to nano-electronics, fuel cells, solar cells, building materials, sensors, gene delivery, drug delivery, tissue engineering, gas storage, microscopy, etc. With advances in material and chemical sciences and availability of methods to prepare/synthesize and characterize these materials, there is a global call for funding nanotechnology research. Nanotechnology is an emerging science involving manipulation of materials at nanometer scale and it offers enormous opportunities and applications in drug delivery and biomedical research. Nanotechnology refers to the structures with one or more dimensions in the 1–100 nm size range. However, nanoparticles (NPs) applied as drug delivery systems are up to several hundred nanometers (<300 nm) in size and are made up of different materials such as polymers (poly(lactic-co-glycolic acid)-PLGA), metal NPs (quantum dots-QDs), and carbon nanotubes (CNTs) etc. [4, 5]. Various nanotechnological approaches are being used to achieve better therapeutic outcome. Application of nanotechnology in medical research and clinical practice for the treatment, diagnosis, monitoring and control of biological systems referred to as "nanomedicine" [6]. Nanomedicines propose solutions to the age old problems associated with the solubility, bioavailability, cellular uptake, and toxicity of many of the classical drugs [7]. Most important areas that seem to have a lot of benefits from nanotechnology is oncology for cancer diagnosis, targeting, therapy and therapeutic monitoring of tumor progression and CNS disorders to deliver therapeutic agents to brain [8–10].

With the discoveries and improvements in nanotechnology and the projected growth rate, it is expected that the production and use of nanomaterials will increase considerably in near future. As the materials become available and their usage increase, the exposure to such materials will increase. Thus, the safety concerns about NP-containing materials are increasing. The NPs show different physicochemical properties when compared to corresponding bulk materials. The small size of NPs confers a large surface area which, in turn, leads to increased interactions with the cells. Thus, in such a scenario, the toxic effects of NPs remain not a function of their mass (as in case of bulk materials) but become a function of the surface area [11].

1. Although there is a tremendous increase in applications of nanoparticles in medical imaging, disease diagnosis and drug delivery, the possible toxic health effects of these nanoparticles associated with human exposure through various routes like inhalation, ingestion, dermal and intended injection have not been properly studied [12, 13]. To date, only few studies are available on the toxicological effects of nanomaterials and no clear guidelines exist to carry out the nanotoxicity studies [14–20]. So there is a great need for the toxicological evaluation of different nanoparticles in order to frame the regulatory guidelines [21].
2. CNTs, due to their unique physicochemical properties and the ability to cross the cell membrane, have become attractive candidates for numerous biomedical applications [22–26]. Both single walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) have been extensively explored for various therapeutic applications [24, 27–30]. Various polymeric NPs, including PLGA NPs have shown a lot of promise in nanotechnology based drug delivery systems [31–33]. Quantum dots (QDs) with unique photophysical properties are found to be suitable for various biomedical applications including drug delivery and diagnostic agents [34, 35]. However, novel and emerging technologies have their own teething problems, and nanotechnology is no exception. The current information on the toxicity of NPs is equivocal as several studies reported on various types of NPs indicate contrasting toxicological outcomes [28, 36–41]. Thus, maximizing the use of nanomedicine is possible only after methodical analysis of the risk versus benefit [42].

   It is strongly believed that NPs interact with the biological systems very strongly when compared to that of bulk material [12]. Nanomaterial safety is a growing concern due the lack of sufficient information and these opinions lead to the origin of concept of “Smaller is not always better: Nanotechnology yields nanotoxicology” [43]. Hence, the emerging discipline of nanotoxicology has come up to address the new potential threats associated with NPs [44]. It has been hypothesized that due to smaller size, NPs can cross all the biological barriers including the Blood–Brain Barrier (BBB) [45–47]. However, the effects of NPs on BBB function and related consequences are not properly studied and indicating the need of nanoparticle toxicity in brain. Thus, new investigations dealing with the effects of several kinds of nanoparticles on the CNS with special regard to BBB function is urgently needed [48, 49].

3. Nanotoxicology research not only provide data for safety evaluation of nanomaterials, but also help to advance the field of nanomedicine by providing information about their undesirable properties and means to avoid the toxic issues by modifications [50]. However, during last 10 years only limited numbers of studies were devoted to evaluate the toxicity of the nanoparticles [20]. Most of the nanotoxicological studies reported so far were based on in vitro cell culture systems [51–53]. Due to the complex biological environment, entirely different effects for the same NPs may be obtained in in vivo studies when compared to in vitro studies. Changed NPs characteristics, when they interact with biological tissues/fluids cannot be interpreted from the in vitro cell culture tests [54]. Therefore, systematic in vivo toxicological studies are needed to ensure the nanomaterials to be suitable for drug delivery and other biomedical applications [55]. Thus, balancing the risk and benefits, maximizes the utility of these materials in nanomedicine without compromising public health and environmental integrity.

23.2 Introduction to Nanotoxicology

The unique physical and chemical properties that make nanomaterials so attractive may be associated with potentially adverse effects. Novel physicochemical properties of the nanomaterials cannot be predicted based on our current understanding of their behavior in larger bulk forms. The specific physicochemical properties of the nanoparticles are expected to result in increased reactivity with biological systems. However, in this era of nano-opportunities, one must not forget to assess any potential threats of nanotechnologies to human health [56]. The fast growing number of applications of engineered nanoparticles in drug delivery systems, medical devices, food products, consumer products and the subsequent disposal of engineered nanoparticles in the environment may increase the human exposure [57]. The emergence of novel materials with new and unanticipated properties has prompted the emergence of a new subcategory of toxicology called nanotoxicology to address the potential threats that widespread use of new nanomaterials could bring [58–60]. Several studies indeed suggest that these nanoparticles have a different toxicity profile compared with larger particles [44]. The safety of the nanoparticles, lead to concept that smaller is not always better: nanotechnology leads nanotoxicology [43] and it is considered that nanotechnology and nanotoxicology are the two sides of the same coin [61]. However, the rapid progress in nanotechnology has exceeded the progress of research on nanotoxicology [62].

The novel materials can be safely applied in a clinical setting, if only their biocompatibility, biodistribution and biodegradation is carefully assessed [63]. The extensively used solution in assessing nanomaterial toxicology is by utilizing various mammalian cells to test for viability or increase/decrease in a designated inherent biological pathway against chosen engineered nanomaterials [64, 65].

Emerging nanotechnologies are unlikely to succeed without appropriate research into understanding and managing potential risks to health, safety, and environment [66].

However, specific mechanisms and pathways through which nanomaterials may exert their toxic effects remain unknown [61]. Nanoparticle toxicity depends on biocompatibility, biodistribution, and biodegradation of the particles inside the body [67].

It has been demonstrated through both in vivo and in vitro studies that the properties that make nanomaterials so attractive from a commercial application point of view can also potentially be responsible for undesirable health effects [44]. Despite current research, significant knowledge gaps exist in all areas of nanotechnology risk assessment. Several laboratories have reported potential toxic effects of nanoparticles on different types of cells in vitro conditions [13, 68]. However, reported in vitro cell culture mediated nanotoxicity studies may not correspond to in vivo results and sometimes NPs may interact with the cell culture medium to influence the outcome of the experiments [13, 69, 70]. There are only a few publications on in vivo toxicity of nanomaterials [71, 72], indicating the need for the more in vivo studies [55]. The pharmacokinetics and biodistribution of the nanoparticles largely define their therapeutic effect and toxicity, which can be done in only animal models [73, 74]. Most of the nanoparticle animal toxicity studies are limited only to respiratory exposure [75–77]. In reported non-respiratory toxicity animal models, the doses used were too low to predict the possible human exposure [78]. Nanotoxicology is a relatively new field and most of the reports have focused on acute toxicity. Therefore, in a toxicological point of view, a large range dose nanoparticle exposure at different exposure period covering acute and chronic exposure needs to be evaluated. Interpretation of in vivo toxicity studies has proven to be challenging as several factors may be working in tandem to cause nanoparticle toxicity [12]. A detailed assessment of the factors that influence the biocompatibility and/or toxicity of nanoparticles is crucial [79].

Nanoparticle toxicity not only depends on particle size but also on other physicochemical parameters such as size, shape, purity, chemistry, solubility, surface properties, aggregation, and stability [80–86]. Although the mechanism underlying NPs induced cytotoxicity is not completely understood, but it has been suggested to be related to inflammatory response, oxidative stress, p53 activation and apoptosis, etc. [18, 84, 87–94].

Gold nanoparticles biodistribution resulted in the wide spread distribution with smallest size (10 nm), whereas the larger particles were only detected in blood, liver, and spleen [95]. The varied biodistribution may result in the different toxicity profile of the nanoparticles with different particle size. In another study after intravenous administration of gold nanoparticles significant biodistribution of Au in the body over 2 months period and accompanying gene expression changes were observed in targeted organs [96]. Liver accumulated with gold nanoparticles resulted in the altered metabolizing CYP enzymes, indicating the need for careful safety evaluations [97]. The mechanism of gold nanoparticle toxicity was reported as autophagy and associated oxidative stress and autophagosome formation [98]. In contrast to QDs toxicity, where toxicity is reduced by surface coating, the silver nanoparticles coated with polysaccharide resulted in the significant increase in the genotoxicity when compared to the uncoated silver nanoparticles on mouse embryonic stem and

embryonic fibroblast cells [99], indicating that surface coating may not always result in the reduced toxicity. In a recent study, transfer of QDs from female pregnant mice to their fetus across the placental barrier has been reported [100]. Nanoparticle induced endothelial toxicity and endothelial dysfunction has been reported with different nanoparticles [101, 102]. Nevertheless, more in vivo experiments are needed to study the different types of characterized nanomaterials with relevant route of administration which closely reflecting expected exposure levels to evaluate the toxicity of nanomaterials [103].

Therefore, proper assessment of the risks associated with exposure to nanomaterials requires a much better fundamental understanding of the impact of these materials on biological systems [104]. The rapid commercialization of nanotechnology requires focused safety assessment to prevent the potential risks associated with nanotechnology [105]. However, to date, no conclusive links between engineered nanoparticles and a biological or health impact have been observed, and insufficient data exists to make generalizations about the biocompatibility or safety of the nanomaterials [106].

Nanotoxicology relies on many analytical methods and techniques for the characterization of nanomaterials as well as their impacts on in vitro and in vivo function [107]. Development of new techniques to study the in vitro and in vivo nanotoxicological studies is essential to accurately represent nanoparticle effects [108]. An integrated metabolomic approach may be useful for the development of a rapid in vivo screening method for nanotoxicity [109, 110]. Therefore, it has been considered that nanotoxicological studies are needed not only to protect human health and the environment, but also to avoid damaging the nanotechnology industry in the longer term [17]. Despite of so many studies conducted on nanoparticle toxicity, the scientific community is yet to determine which nanomaterials are hazardous to the environment or humans [57, 111]. From the regulatory point of view it is very difficult to make any general regulatory recommendations to conduct the risk assessment due to still limited safety data of nanomedicine [112]. It is recommended that every nanomedical applications be evaluated based on a case-by-case approach until specific guidelines can be finalized [42]. The present challenge for the nanotoxicologist is to identify key factors to predict the nanomaterial toxicity and how to improve the toxicity by rational modifications and predictive toxicological paradigm for the safety assessment of nanoparticles has been recently proposed based on the oxidative stress model [113–115].

23.3 Carbon Nanotubes

Carbon nanotubes (CNTs) consist exclusively of carbon atoms often described as a graphene sheet rolled up into the shape of a cylinder. This novel nanomaterial belongs to the fullerene family, the third allotropic form of carbon along with graphite and diamond. CNTs can be classified into single walled carbon nanotubes (SWCNTs)-consist of one layer of cylinder graphene and multiwalled carbon

nanotubes (MWCNTs)-contain several concentric graphene sheets. SWCNTs have diameters from 0.4 to 2.0 nm and lengths in up to few microns (μ), while MWCNTs diameters in the range of 1.4–100 nm and lengths up to few microns [28]. CNTs have fascinated scientists with their extraordinary physicochemical properties. These materials have become increasingly popular in various fields simply because of their small size and amazing optical, electric, and magnetic properties. CNTs exhibit exceptional mechanical properties (high tensile strengths, light weight, thermal and chemical stability), which results in a number of potential applications in nanotechnology and in nanomedicine as biosensors, diagnostic agents, therapeutic devices, and drug delivery systems [116, 117]. CNTs are ideal nonbiodegradable materials; these are considered to be one of the least biodegradable man-made materials.

Unmodified CNTs were called pristine CNT which are not routinely used for the drug delivery. Different functionalization strategies have been reported for various nanotechnological applications [22]. Functionalization and bioconjugation of CNTs for various biomedical applications have been reported [118, 119]. Functionalized CNTs (F-CNTs) enable variety of medicinal applications, including the diagnosis and treatment of cancer, infectious diseases, and central nervous system disorders and also useful in tissue engineering [26, 120]. F-CNTs are emerging as a new family of nanocarriers for the delivery of different types of therapeutic molecules [27, 121]. One key advantage of CNTs is their ability to translocate through plasma membrane, allowing them for the delivery of therapeutically active molecules [122]. CNTs based nano platform has been developed to deliver various agents into cells, including drugs, peptides, proteins, plasmid DNA, and small interfering RNA [29, 123–126]. CNTs may afford a more effective and convenient delivery system compared to the conventional systems [127].

Functionalization renders the surface of CNTs water soluble, compatible with biological fluids and leads to their rapid excretion through the renal route and minimizing unwanted tissue accumulation [28] and thereby can be developed as nanomedicines.

CNTs owing to their unique physicochemical properties have become a popular tool in cancer diagnosis and therapy [128, 129]. These are considered one of the most promising nanomaterials with the capability of both detecting the cancerous cells and delivering drugs or small therapeutic molecules to these cells [130]. CNTs attached to antibodies or peptides represent important approach to targeting cancer cells [131]. Targeted and controlled delivery of various anticancer drugs with superior anticancer cancer activity has been demonstrated with CNTs [25, 132, 133].

Biological systems are highly transparent to 700–1,100 nm near infrared (NIR) light [134]. The strong optical absorbance of SWCNTs in the NIR region can be used for optical stimulation of CNTs inside living cells. CNTs convert absorbed NIR light into heat, which can thermally ablate cells that have bound the CNTs [135]. Selective destruction of cancer cells with multifunctional SWCNTs containing targeting ligands (folate, RGD peptide, and EGFR antagonist), anticancer agents, and the imaging agents is proposed and few studies have already demonstrated the multifunctional performance of CNTs [23, 136–138]. Rapid photothermal intracellular

drug delivery using MWCNTs was reported, where NIR irradiation of MWCNTs resulting in the increased intracellular delivery of anticancer agents has been achieved [46].

Selective cancer cell destruction was achieved by functionalization of SWCNTs with a folate moiety. Thus, the transporting capabilities of CNTs combined with suitable functionalization chemistry and their intrinsic optical properties can lead to new classes of novel nanomaterials for drug delivery and cancer therapy [23, 139]. SWCNTs show an intense Raman peak produced by the strong electron photon coupling that causes efficient excitation of tangential vibration in the nanotubes quasi one-dimensional structure upon light exposure. The robust Raman scattering property of SWCNTs can be used for the detection and in vivo imaging purpose [78]. Detection of Raman NPs in both superficial and deep tissues will be useful for targeting and imaging of tumors suggesting that CNTs may become promising molecular imaging agents for living subjects [140, 141].

Even though significant benefits have been proposed and demonstrated in various experimental condition for promising therapeutic drug delivery and biomedical applications based on CNTs [142]. Much remains to be done before this can be a clinical reality and many factors need to be optimized, among which are biocompatibility, pharmacokinetics, biodistribution, in vivo targeting efficacy, the ability to escape the RES, and toxicity issues [143]. The toxicological and pharmacological profile of such CNTs mediated drug delivery systems have to be determined prior to any clinical studies.

23.4 Carbon Nanotubes Toxicity

CNTs have produced unusual toxicological properties owing to their unique physicochemical properties [144]. The present knowledge on toxic effects of CNTs is still limited, in spite of the large number of ongoing studies [145]. Comparing the different studies is difficult because different CNTs types (MWCNTs and SWCNTs, difference in diameter, length, size, and surface area and functionalization, etc.) and model systems employed for the toxicological evaluation [146]. The exact methodology and CNTs characteristics are not described, which makes it difficult to compare the data. Furthermore, CNTs contain contaminants which further complicates the comparison of different carbon nanotubes toxicity studies [147]. The toxicity of CNTs has been demonstrated in vitro and in vivo. In general, in vivo administration of small doses of CNTs (<10 mg/kg) was accompanied by accompanied by inflammation and cell death. The in vitro studies complement the in vivo studies and show cytotoxicity and pro-inflammatory potential of CNTs as observed in vivo. CNTs exhibit toxicity towards a variety of cell types; the toxicity being dependent on the CNT type and the cell type. However, in most of the primary cells and cells studied, cells were susceptible to cytotoxic effects of CNTs at concentrations of 100 μg/ml or lower.

23.4.1 CNTs In Vitro Toxicity Studies

Most of the CNT toxicity data and mechanisms of its toxicity was explained with in vitro cell culture experiments by using wide range of cell types including lung fibroblasts, keratinocytes, Hela liver cells, kidney cells, HUVEC cells, macrophages, microglial cells, etc. In vitro studies indicated a decrease in cell proliferation and cytotoxic effects associated with cell death [148, 149], induction of apoptosis or reactive oxygen species generation [150]. Conversely, other authors reported low or no cytotoxic and lack of toxicity in different cell types after treatment with CNTs. Functionalization of CNTs by the addition of certain surface molecule groups can modify their toxicity [151].

Exposure of human keratinocytes mimicking potential dermal exposure has shown that both SWCNT and MWCNT are capable of localizing within cells and causing cellular toxicity [148]. Manna et al. demonstrated a clear dose-dependent activation of NF-κB and oxidative stress in human keratinocytes along with IkB depletion and MAPK phosphorylation [150]. Human T lymphocytes a possible target cell for CNTs that gain access to lymphoid tissue were exposed to oxidized MWCNT and found to be killed in a time- and dose-dependent manner via an apoptotic mechanism [152]. Similarly, other studies also reported the CNTs induced apoptosis [149, 153, 154]. CNT toxicity was also found to be mediated through oxidative stress [90, 155, 156]. However, in vitro nanoparticle toxicity study sometimes may lead to false positive or false negative endpoints. Inflammatory cell mediated toxicity, NP ADME, and long term toxicity studies are not possible with in vitro cell culture experiments [13, 55].

23.4.2 CNTs In Vivo Toxicity Studies

In vivo toxicity studies are essential to prove the safety of any drug delivery systems. Pulmonary toxicity of CNTs has attracted much attention in recent years due to the occupational and environmental significance of these materials [157, 158]. However, due to the difficulties associated with generating aerosols of CNTs, several in vivo pulmonary studies have employed intratracheal instillation as the exposure method to study pulmonary toxicity [77, 88]. SWCNTs instilled into the lungs of mice produced granulomas in the pulmonary interstitium of the lungs [76]. Rats exposed to SWCNTs developed multifocal granulomas in the absence of any pulmonary inflammation or cellular proliferation [159]. Pulmonary toxicity studies of SWCNTs after pharyngeal aspiration in C57BL/6 mice have demonstrated a dose-dependent augmentation of biomarkers of cell injury, oxidative stress as evidenced by reduced level of GSH and total antioxidant reserve along with the accumulation of lipid peroxidation products in Bronchoalveolar lavage (BAL) fluid and in the lung [144]. A subsequent study using C57BL/6 mice that were maintained on vitamin E-sufficient or vitamin E-deficient diets further emphasized the importance of

oxidative stress and antioxidant depletion in the overall inflammatory response in the toxicity of SWCNTs [160]. Muller et al. exposed rats intratracheally to whole or ground MWCNTs and characterized their biological activity. They also measured biopersistence at two time points and found that the longer ungrounded nanotubes were more biopersistent than the shorter nanotubes at 60 days and were highly fibrogenic and inflammogenic [161]. Another study revealed that even after metals are removed SWCNTs induced transient oxidative stress [144]. In a recent study biodegradation of SWCNTs by human neutrophils enzyme myeloperoxidase was reported in the in vitro conditions; further in vivo administration of these degraded CNTs resulted in significant reduction in pulmonary inflammation. These findings indicate the ways to mitigate the CNT toxicity. However, in case of high dose administration of CNTs, the neutrophilic enzyme biodegradation system may be compromised leading to pulmonary toxicity [162]. Oral administration of SWCNTs to rats resulted in the oxidative stress mediated DNA damage in liver and lung cells, raising the concerns when these compounds are used for various biomedical applications but in few other studies the oral administration of CNTs did not result in the appreciable toxicity [163, 164]. In vivo CNT toxicity studies reported till today are mainly focused on pulmonary toxicity to mimic the environmental and occupation exposure. However, the other routes of human exposure of CNTs particularly for intravenous and oral administrations for medical use were mostly overlooked and often confined to low dose toxicity studies with minimal toxicological end parameters. Therefore, there is a need for the more extensive studies involving more factors to determine the possible toxic effects associated with CNTs exposure. Recent in vivo studies have provided encouraging results in terms of biodistribution and biocompatibility of functionalized CNTs, as no acute toxicity or adverse reaction was seen, and rapid excretion of CNTs through the renal route was observed [41, 165, 166]. Functionalization of CNTs may thus serve to minimize adverse biological effects related to unwanted tissue accumulation of this important class of nanomaterials.

Intravenous administration of functionalized SWCNTs in high doses resulted in the accumulation of the CNTs in liver and spleen without any notable toxicity [40]. In an another study, intravenous administrations of high doses of MWCNTs resulted in liver toxicity; further PEGylation partly decreased the liver toxicity in mice, which implies the role of CNTs surface on the toxicity [167]. CNTs were shown to produce length dependent inflammogenic effects at the mesothelial surface similar to asbestos. Follow-up studies in the more relevant pleural mesothelium have shown the intense inflammation being produced by long asbestos and long nanotubes in contrast to insignificant levels of inflammation caused by short asbestos and short nanotubes [168]. Therefore, length dependent CNT toxicity needs to be further studied to find the suitable length for drug delivery purpose. Intravenous administration of purified SWCNTs in ICR mice resulted in the long term accumulation in the liver, lung, and spleen. However, these accumulations resulted in only low level of toxicity which is mediated through oxidative stress [169]. Platelet induction and vascular thrombosis was observed with carbon nanoparticle on in vitro and in vivo test systems and these platelet induction was found to be mediated through influx of

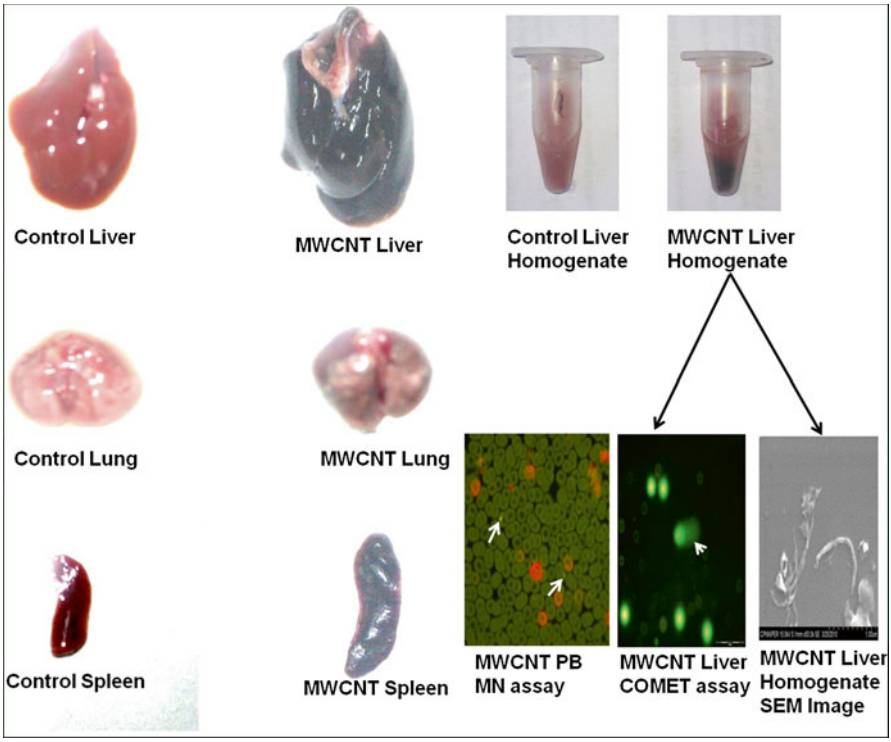

Fig. 23.1 Toxicological effects of MWCNTs: Mice were intravenously administered with MWCNTs at the dose of 10 mg/kg (single dose), 1 month after the administration, the accumulation of the MWCNTS were seen in liver, lung, and spleen. The black colored organs indicate the accumulation of CNTs. Organ weights were also increased in MWCNTs treated groups (data not shown). In our follow-up studies similar kind of accumulation was seen even 3 months after the single dose of MWCNTs (10 mg/kg) administration, which suggested the long term accumulation property of MWCNTs in reticuloendothelial system (RES) organs. Similarly, 24 h after the MWCNTs administration, significant increase in the micronucleus (MN) formation was observed in peripheral blood smears, *white arrows* indicate the MN in the erythrocytes. COMET assay performed in the liver cells collected from MWCNTs showed marked increase in the percentage DNA in the tail which is the indicator of frequency of DNA damaged (*white arrow head* indicate the moment of DNA in damaged cells). Both MN and COMET assays demonstrated the genotoxic potential of MWCNTs. Upon acid digestion of liver collected from 3 months post MWCNTs treated animals, the acid digested matrixes were visualized under scanning electron microscope (SEM), the presence of intact MWCNTs indicate the long term accumulation behavior of MWCNTs in animal models. These data clearly indicate the toxic potential of MWCNTS (unpublished data)

extracellular calcium [16, 170, 171]. Based on the contrasting toxicological outcomes with different types of CNTs in different animal models and on top of it limited number of toxicological studies, in future detailed in vivo toxicological studies are needed to ensure the safety of the CNTs for drug delivery purpose (Fig. 23.1).

Further, pulmonary exposure to MWCNTs also resulted in increased coronary vasoconstriction or reduction in endothelium-dependent vasodilation [172, 173].

SWCNTs were found to show toxicity in rat heart cell line. Light microscopy revealed changes in cellular morphology with slight reduction in cell viability and increase in number of apoptotic cells. When subcultured, SWCNT-treated cells showed higher degree of cell death with an increase in apoptotic cell density. The cells regained partially reverted to their normal morphology and divided normally [174]. Contrastingly, SWCNTs also promoted transformation to myofibroblasts [175] while cardiac myocytes cultured on MWCNT supports showed higher viability [176]. Further, incubation with SWCNTs resulted in an increased expression of cell adhesion molecules, oxidative stress, and apoptosis in rat aorta endothelial cells [177–179]. Intravenous administration of MWCNTs in rats resulted in increased calcification of aorta as well as damage to aortic epithelium [180]. In vitro studies suggested the MWCNTs may accelerate progression of atherosclerosis [181]. Similarly, pharyngeal aspiration of SWCNTs and MWCNTs resulted in alterations in expression of several genes in aorta. An increased expression of E-selectin, P-selectin, IL-6 (pro-inflammatory cytokine), and HO-1 (oxidative stress marker) was observed in aorta. Intratracheal instillation of SWCNTs in spontaneously hypertensive rats resulted in an increase in plasma levels of angiotensin-I converting enzyme suggestive of peripheral vascular thrombosis [182]. Oropharyngeal aspiration of MWCNTs in mice resulted in worsening of cardiac ischemia–reperfusion injury in a dose- and time-dependent manner [183].

The pulmonary exposure of NPs may also lead to effects on the central nervous system (CNS) [184, 185]. These central effects of NPs may arise due to retrograde axonal transport through neurons. Apart from these, NPs in systemic circulation may also cross blood–brain barrier and exert CNT toxicity. SWCNTs showed time- and concentration-dependent cytotoxicity in a neuronal-like cell line, PC12. The cytotoxicity was accompanied by stimulation of free radical production and induction of apoptosis [186, 187]. Functionalization of SWCNTs with PEG resulted in a reduction in oxidative stress [188]. Further, incubation with Vitamin E resulted in reduction in SWCNT-induced oxidative stress and apoptosis in PC12 cells [189]. MWCNTs have been demonstrated to suppress potassium channels and increased neuronal differentiation in PC 12 cells [190, 191]. MWCNTs also altered excitability in chromaffin cells [192], rat brain slices [193], and hippocampal CA1 neurons [194]. MWCNTs have been demonstrated to be cytotoxic in dorsal root ganglia neuron cultures and inhibited axon growth [195, 196]. Similarly, MWCNTs were also found to be toxic towards rat glioma cells and induced oxidative stress [197]. Amino-functionalized MWCNTs increased neurite outgrowth in cultured PC12 and dorsal root ganglia neurons [198]. When administered to pregnant dams, the pups did not show any change in neurobehavioral parameters [199].

Schipper et al. assessed the toxicity of intravenously administered PEG-functionalized SWCNTs in nude mice. The animals were observed for 4 months and showed no significant changes in body weight, blood cell counts (erythrocytes, leucocytes, neutrophils), hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration as compared

to control. The CNTs were observed in lungs and spleen by histopathology and Raman microscopy without evidence of toxicity [40]. The PEG-SWCNTs were found to be excreted in feces [200]. Wang et al. demonstrated induction of Th2 response in splenic T cells of mice intravenously injected with MWCNTs [201]. Pristine and oxidized MWCNTs were found to induce inflammatory response and hyperalgesia in animals subcutaneously injected with CNTs [202–204].

23.5 Fullerene Toxicity

Due to putative antioxidant properties Fullerenes C60 have a lot of therapeutic applications. Assessment of toxicity associated with C60 fullerene preparations is of pivotal importance for their biomedical application as cytoprotective (antioxidant), cytotoxic anticancer, theragnostic or drug delivery agents. Moreover, the widespread industrial utilization of fullerenes may also have implications for human health [205]. In another study, adult zebrafish exposed to the 30 mg/kg dose fullerene for 24 h have shown enhanced Acetylcholine esterase (AChE) activity and increased oxidative stress (increased lipid peroxidation levels) in brain, suggesting the possibility of fullerene nanoparticles crossing the blood–brain barrier and reaching the brain and causing toxicity [206]. Co-exposure of Fullerene with carcinogenic toxicant benzo[a]pyrene (BaP) to zebrafish hepatocytes resulted in aggravation of BaP toxicity through increasing intake of BaP into cells, decreased cell viability and impaired detoxifying phase II enzymes such as GST. This observation suggest that in presence of Fullerene, there may be a possibility of increased environmental toxicant induced carcinogenic effect [207]. Further, in ischemia reperfusion injury model intratracheal and systemic administration of fullerene resulted in increased myocardial infarction in coronary arteries. In vitro tissue responses indicated the increased endothelin-1 mediated vasoconstriction in coronary arteries. This increased contraction was augmented with indomethacin, suggesting the role of COX-2 pathway in vascular dysfunction associated with fullerene. The acetylcholine induced endothelium dependent vascular relaxation was significantly reduced in fullerene intravenously administered male rats, where as in IT C60 exposure groups decreased sodium nitroprusside mediated vasorelaxation was observed in female rats. Intravenous administration of fullerene resulted in increased serum interleukin-6 and monocyte chemotactic protein-1. Based on these observations, it was concluded that intratracheal and intravenous exposure of C60 results in unique cardiovascular consequences that may favor heightened coronary resistance and myocardial susceptibility to ischemia reperfusion injury [208]. In an air pouch model of acute inflammation, fullerene alone did not induce any kind of inflammation and proinflammatory cytokine release; however, fullerene can act in concert with other agents to cause inflammation; in the presence of lipopolysaccharides (LPS) fullerene increased the local production of several cytokines/chemokines, similar type of situation that is likely to occur in vivo [209].

23.6 Polymeric Nanoparticles Toxicity

Polymeric Nanoparticles are solid colloidal particles composed of natural, synthetic, or semisynthetic polymers with size ranges from 1 to 1,000 nm. Synthetic biodegradable polymers are well-known materials for the synthesis of nanoparticles (NPs) [210, 211]. The drugs or other bioactive molecules can be loaded into the nanoparticles by entrapment or encapsulation or adsorption or attachment methods [212]. Many studies have reported to deliver a variety of drugs such as hydrophilic drugs, hydrophobic drugs, proteins, vaccines, and biological macromolecules using polymeric nanoparticles as carriers [213, 214].

Polymeric nanoparticles are widely investigated for controlled and targeted delivery of various drugs. Although a number of different polymers have been investigated for formulating biodegradable NPs, PLGA polymer has been the most studied [215]. Among all the polymers, copolymer Poly(lactide-co-glycolide) (PLGA) is approved by the US Food and Drug Administration, has attracted significant interest for nanoparticle delivery system because it is biodegradable, biocompatible, nontoxic and generally regarded as a safe (GRAS) polymer [216]. PLGA has been widely applied to formulate hydrophobic as well as hydrophilic drugs [216]. PLGA nanoparticles have been extensively used as carriers for drug delivery and other therapeutic applications and also as multifunctional delivery system [217, 218].

There are several techniques available for the preparation of drug loaded PLGA NPs for various therapeutic and biomedical applications [219, 220]. Among all, the solvent evaporation technique is often adopted due to its high drug loading capacity, good reproducibility, high stability, and applicability to both hydrophobic (the single emulsion method) and the hydrophilic drugs (the double emulsion method). Several other polymers such as chitosan, dendrimers, block polymers, PBCA have also been demonstrated in nanomedicine to improve the performance of the therapeutic agents [212, 221–225]. Though studies on polymer toxicity in bulk form are available, due to unexpected physicochemical characteristics and increased reactive nature of the nanoparticles, detailed nanotoxicity studies are needed to ensure the safety of the polymeric nanoparticles [226–228].

23.7 Quantum Dots Toxicity

Quantum dots (QDs) are luminescent semiconductor nanocrystals with approximate size 2–100 nm in diameter. They have advanced optical properties compared with traditional organic fluorophores [229]. They have size and composition tunable emission from visible to infrared wavelengths, broader excitation spectra due to high absorption coefficients, high quantum yield of fluorescence, strong brightness, photostability, and high resistance to photobleaching [230]. These unique properties of QDs have attracted tremendous interest in exploiting them in a variety of biological

applications [231]. QDs comprise metallic components of cadmium-selenium (CdSe) or cadmium-tellurium (CdTe) cores with zinc sulfide (ZnS) shells. They have the great potential to become one of the most useful tools for biomedical applications [232]. The potential applications of QDs are to use as optimal fluorophores for in vivo biomedical imaging [149, 233, 234]. QDs have also been proposed for biological labeling or cell targeting (e.g., labeling cancer cells) following conjugation with specific bioactive moieties [235, 236]. They were intensively explored as fluorescent probes for long term and multimodal in vitro and in vivo imaging purposes [35, 237, 238]. Targeted delivery of QDs can also achieve high specificity and sensitivity of cancer detections, which can diagnose cancer at its earliest stage and thus greatly improve its prognosis [239, 240]. Despite the immense potential of the QDs for clinical and research applications, the potential applications were limited due to the toxic effects of semiconductor QDs. Their biological effects and safety are still unclear, which have received enormous attention over the past few years [62, 241].

With the widespread use, the likelihood of exposure to QDs has been assumed to have increased substantially. However, there is a lack of toxicological data pertaining to QDs [242]. All QDs are not alike, engineered QDs developed for various applications cannot be considered a uniform group of substances. Their absorption, distribution, metabolism, and excretion (ADME) and toxicity depend on multiple factors such as physicochemical properties and environmental conditions [243]. QDs size, charge, concentration, outer coating, and mechanical stability have each been implicated as determining factors in QD toxicity [82, 243, 244]. Although they offer potentially invaluable benefits such as drug targeting and in vivo biomedical imaging, QDs may also pose risks to human health and the environment under certain conditions. Recently published research indicates that there is a range of concentrations where quantum dots used in bioimaging have the potential to decrease cell viability or even cause cell death, thus suggesting that further toxicological evaluation is urgently needed [245]. Most of the QDs toxicology data is derived from in vitro experiments and these studies may not reflect in vivo responses [246].

To date, the literature on toxicity of QDs is combination of several types of QDs with widely varying physicochemical parameters, making the comparisons rather difficult [247]. A primary source of QDs toxicity results from cadmium residing in the QDs core. Toxicity of uncoated core CdSe or CdTe QD have been discussed in several reports and is associated, in part, with free cadmium present in the particle suspensions or released from the particle core intracellularly [248]. Encapsulation of the CdSe QDs with a ZnS shell or other capping material has been shown to reduce toxicity [249]. Intravenous administration of QDs (CdSeTe) in mice resulted in the persistent accumulation in the spleen, liver, and kidneys for at least 28 days with little or no disposition, but was gradually and partially eliminated by 6 months. Histological examination of these organs by normal light microscopy did not result remarkable alteration. But, investigations using electron microscopy on renal samples revealed definitive mitochondrial alterations in renal tubular epithelial cells at 28 days and 6 months post dosing indicate the possible toxicity [241]. Systemically administered QDs persisted with significant blue-shifted emission and retained fluorescence for up to 2 years in vivo [250]. At higher doses QDs were found to

produce pulmonary vascular thrombosis by activating the coagulation cascade, these effects depend on the surface charge of the QDs, emphasizes that surface charge is an important parameter in assessing QDs nanotoxicity [251]. In an another study low dose 5 nM/mice of QDs administration resulted in the significant clearance from the body mainly from kidney renal filtration and from liver hepatic biliary excretion and little fraction (8 % of the dose) of QDs were found to be accumulated in liver for longer periods [229]. Short term and long term toxicological evaluation of CdSe QDs dots in healthy Sprague Dawley at 2.5–15 nmol dose did not cause appreciable toxicity even after breakdown in vivo over time [246].

Cytotoxicity and proinflammatory cytokine induction of QDs on Human epidermal keratinocytes (HEKs) indicate that QD surface coating is a primary determinant in QDs toxicity [82]. When compared to CdSeTe core, QDs containing $CuInS_2$ as a core resulted in the improved toxicity in animal models [252]. QDs treatment resulted in the increased production of tumor necrosis factor (TNF)-α and CXC-chemokine ligand (CXCL) 8 levels through ROS and mitogen-activated protein kinases (MAPKs) dependent mechanisms in human primary monocytes and in mice models [15]. Based on the limited toxicological data, the potential toxic effects of QDs have become a hot issue that must be further addressed before clinical applications would be possible. Most studies recommend that not all QDs are similar in their toxicity, and toxicity of differing QDs must be considered individually. The adverse effects of QD can be mitigated or eliminated by proper choices of coating materials and modification techniques that improve the stability [12].

23.8 Cerium Oxide Nanoparticles (Nanoceria) Toxicity

The vascular infusion of nanoceria resulted in acute sequestration of liver Kupffer cells and subsequent retention on parenchymal cells. Hepatic stellate cells also sequestered nanoceria. Within the sinusoids, prolonged nanoceria bioretention was associated with Kupffer rich granuloma formation. The intermingling CD3+ T cells were also observed in granuloma. Upon acute exposure significant increase in the serum aspartate aminotransferase (AST) level was seen, this elevation was subsided in chronic infusion conditions. In liver tissue increased protein carbonyl levels were observed, indicating the role of oxidative stress in chronic nanoceria induced liver injury. These observations suggest a single vascular infusion of nanoceria can lead to persistent hepatic retention of particles with possible implications for occupational and therapeutic exposures [253].

23.9 Genotoxicity of Nanoparticles: Nanogenotoxicity

One of the important areas governing regulatory health risk assessment of NPs is genotoxicity, as DNA damage may initiate and promote carcinogenesis. Considerable attention has been given to the toxicity of engineered nanomaterials, but the

importance of NPs genotoxic potential on human health has been largely ignored [17]. Many of the engineered nanomaterials assessed were found to cause genotoxic responses, such as chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles. Very little has been done with respect to investigate the genotoxicological safety of nanomaterials used in drug delivery applications [254]. However, due to inconsistencies in the literature, it is difficult to draw conclusions on the features of nanomaterials that promote genotoxicity. Thus, further attention to improve our understanding of the genotoxic potential of engineered nanomaterials is needed [17].

These unique physicochemical properties of NPs may lead to dramatic change in biological activities including ROS generation and inflammation, two of the most frequently reported NPs-associated toxicities. Oxidative stress induced by NPs can be either originated from acellular factors such as particle surface, size, composition, and presence of metals or cellular responses such as mitochondrial respiration, NPs–cell interactions, and immune cell activation are responsible for ROS-mediated damage. NPs induced oxidative stress play pivotal role in further pathophysiological effects including genotoxicity, inflammation, and fibrosis by activating associated cell signaling pathways [255].

Upon interaction of NPs with cells may result in the formation of reactive oxygen species (ROS). At low levels ROS production, cells initiate a protective antioxidant defense mechanism to maintain pro-oxidant and antioxidant balance, which promotes cell survival. An imbalance toward the pro-oxidative state is often referred to as oxidative stress. Under conditions of excess ROS production, the natural antioxidant defenses may be overwhelmed and at higher levels of ROS the protective response is overtaken by inflammation and cytotoxicity. Inflammation is initiated through the activation of pro-inflammatory signalling cascades, such as mitogen-activated protein kinase (MAPK) and nuclear factor κB (NFκB). An excess of ROS can cause apoptosis. Activated transcription factor NFκB stimulates the production of inflammatory cytokines (example tumor necrosis factor alpha (TNF-α), interleukins, IL). The release of cytokines has the potential to cause inflammation under in vivo conditions and can ultimately promote the development of diseases (fibrosis, cancer, apoptosis, and necrosis). Thus, increased levels of ROS either from NPs surface or through NPs-cellular interactions can cause genotoxicity [256]. Genotoxicity of NPs varied highly among different types of nanoparticles [257]. Many reports are available on the negative genotoxic potential of various types of nanoparticles [258–261]. Several in vitro and in vivo studies with carbon nanotubes resulted in the negative genotoxic effects [262, 263]. On other hand, in vitro studies on mouse embryonic stem cells with MWCNTs demonstrated the potential of genotoxicity induction and indicated the need of careful scrutiny of the nanomaterials genotoxicity [264].

The stable and well characterized C60 fullerene nanoparticles did not show any genotoxicity upon in vitro and in vivo assays [261]. In another study stable aqueous suspensions of C60 fullerenes free of toxic organic solvents on human lymphocytes by using single cell gel electrophoresis (COMET) assay has demonstrated positive genotoxic response with strong correlation between the genotoxic response and C60 concentration [265].

Size dependent entry of SiO_2 nanoparticles into the nucleus and disturbance of nuclear organization indicates the direct effect of nanoparticles on the nucleus [266]. The transmission electron microscopic (TEM) analysis indicated the presence of silver nanoparticles inside the mitochondria and nucleus. Disruption of the mitochondrial respiratory chain by accumulated NPs may lead to production of ROS and interruption of energy synthesis, which in turn may cause DNA damage. Further, DNA damage may be augmented by deposited NPs in the nucleus [267]. Strong binding between DNA and Carbon NPs in a dose-dependent manner was observed, which may induce DNA aggregation and DNA damage [268]. The direct binding of nanoparticles with DNA may explain the altered gene expressions upon NPs exposure observed in various studies.

Nanoparticles through increased free radical generation and depleted endogenous antioxidant levels produced genotoxicity in various studies [163, 269, 270]. Coated Ag NPs exhibited more severe damage than uncoated NPs, suggesting that nanoparticle surface play role in the genotoxicity [99]. The genotoxic potential of SWCNTs on lung fibroblast cells by comet assay and micronucleus (MN) test indicated the positive genotoxic response, whereas in the Salmonella gene mutation assay, no mutations were found following SWCNT exposure, indicating the need of multiple genotoxic tests to screen the nanoparticles [271]. In vivo micronucleus (MN) assay in type II pneumocytes 3 days after the single intratracheal administration of MWCNTs and in vitro cytokinesis-block micronucleus assay in rat lung epithelial cells exposed to MWCNTs, both in vitro and in vivo tests indicated the positive genotoxicity. Further, fluorescent in situ hybridization assay evidenced the clastogenic as well as aneugenic effect [272]. In another study treating the mice with TiO_2 NPs in drinking water resulted in the significant DNA damage studied by set of genotoxicity tests such as comet assay, micronucleus assay, gamma-H2AX immunostaining assay and 8-OHdG levels estimations. These results described NPs induced genotoxicity in vivo in mice possibly caused by a secondary genotoxic mechanism associated with inflammation. Another in vitro study demonstrated that exposure to nano TiO_2 disturbs cell cycle progression and duplicated genome segregation, leading to chromosomal instability and cell transformation [273]. Given the growing use of TiO_2 nanoparticles, these findings raise concern about potential health hazards associated with TiO_2 nanoparticles exposure [274]. In contrast, other studies reported the lack of genotoxicity by titanium dioxide nanoparticles [275]. Similarly carbon nanotubes, fullerene and other NPs genotoxicity studies resulted in the contrasting outcomes [259, 265, 276]. Genotoxic, mutagenic or carcinogenic effects of different types of NPs (other than metal nanoparticles) in different animal species are limited.

It is clearly not known whether nanoparticles directly interact with DNA or indirectly effects through inflammation mediated oxidative stress to produce genotoxicity. Mechanisms of genotoxic effects of nanoparticles can be diverse and their elucidation can be demanding. Recognizing the different ways by which various NPs interact with genetic material will improve the possibility for an optimal choice of tests and test conditions for extrapolations of genotoxicity test results to human

risk. Thus, the use of a battery of standard genotoxicity testing methods covering a wide range of mechanisms will be appropriate to study the nanogenotoxicity [254].

DNA damage and chromosomal aberrations are believed to be the prime factors resulting in cell cycle arrest. The cells which successfully repair the damage will reenter the cell cycle, and those with massive damage will not be able to repair the DNA effectively and undergo apoptosis [267]. The DNA damaging agents have the potential to cause genome instability, which is a predisposing factor for carcinogenesis. Hence, it is very important that the biological applications employing nanoparticles should be given special attention for genotoxicity along with their potential biomedical applications.

23.10 Nanoparticles on Blood–Brain Barrier

The blood–brain barrier (BBB) is a specialized system that protects the brain from the penetration of xenobiotics. It consists of endothelial cells connected by complex tight junctions. Generally, most molecules cannot cross the BBB. NPs made of different materials could cross the BBB [277]. The BBB is the most important limiting factor for the development of new drugs and drug delivery systems for the central nervous system (CNS) [278]. With unprecedented increase in the population afflicted by neurodegenerative disorders, it has become increasingly important to develop a dosage form capable of surmounting the challenges imposed by the anatomical barrier of the brain [279, 280]. Nanotechnology plays a unique role in the development of brain specific drug delivery, imaging, and diagnosis [281–284]. A successful strategy to increase drug penetration into the brain is the use of polymeric nanoparticles [285]. Owing to their special properties, NPs have the capacity to bypass the BBB. There are two possible pathways for nanoparticles to reach the brain. The first pathway is mediated though the uptake of nanoparticles by sensory nerve endings and olfactory bulbs present in the airway epithelia [286, 287]. Another pathway is the uptake through the BBB via systemic circulation. This route of brain distribution was largely explored for nanoparticle mediated therapeutic delivery of agents to treat various disease conditions, particularly brain tumors and neurodegenerative disorders [282, 288, 289]. Further, coating of the NPs with the polysorbate (Tween 80) surfactants improved the transportation of drugs across the BBB [290–295]. By modifying the surface, NPs could deliver drugs of interest through the BBB for diagnostic and therapeutic applications in neurological disorders, such as Alzheimer's disease [296]. Coating of nanoparticle surfaces with apolipoprotein E (plasma protein) makes them to break the blood–brain barrier (uptake by endothelial cells via the LDL receptor and transcytosis of the particles across the barrier) is one recent example of this concept [297]. It is evidenced that apo-E represents an essential factor in the transport of low density lipoproteins (LDL) through the BBB because of the great presence of LDL receptors on the BBB cell membranes [293]. Tween 80-coated nanoparticles mimic lipoproteins after apo-E adsorption on their

surfaces and undergo endocytosis through LDL receptors in endothelial cells [294]. Specific ligands or antibodies on the surface allow the NPs to cross the BBB [298]. Antioxidant CoQ10 loaded PLGA nanoparticles resulted in the increased brain uptake and improved the Alzheimer's symptoms in animal models [33]. Several therapeutic drugs were successfully delivered to brain by nanoparticulate system in experimental conditions [293, 299, 300].

However, one of the great concerns in nanotechnology field is that NPs may produce potential toxicity effects on human neural cells due to their ability to pass through biological barriers. Owing to their special physicochemical properties, NPs may cause neurotoxicity after entering into the brain. They may subsequently influence the BBB function and brain physiology and cause severe side effects [301, 302].

Therefore, the evaluation of the potential neurotoxic effects of the NPs on CNS function is urgently needed. The reports on NPs brain penetration and toxic effects on CNS are still lacking and often contradictory in nature. Nanoparticles prepared from different materials result in the varied brain distribution and the most important parameter controlling the NPs' brain distribution is the size of the NPs. The current knowledge on NPs interactions with the CNS system is extremely limited and traditional drug toxicology studies may not be ideal models to draw comparison with NPs [303]. Thus, neurotoxicity induced by NPs is still a new topic that requires more attention [49]. Small size NPs may across the BBB either by passive diffusion or by carrier-mediated endocytosis and accumulate in different regions of the brain. This may be beneficial for drug delivery, but may also pose a risk to the patient when the target delivery of the drug is not brain. Maternal exposure of mice to TiO_2 NPs resulted in the altered expression of genes related to the development and function of the CNS of newborn pups. The genes associated to oxidative stress and apoptosis were changed in the brains of new born mice in early age and induced inflammation and neurotransmitters in the later stage [304]. Nasal instillation of TiO_2 NPs managed to translocate into the CNS and cause potential lesions in brain. Increased oxidative stress such as lipid peroxidation, protein oxidation, and increased activities of catalase, as well as the excessive release of glutamic acid and nitric oxide were observed [185]. Daily for 14 days intraperitoneal administration of TiO_2 NPs resulted in the accumulation in the mouse brain, which caused the oxidative stress and brain injury [47]. A significant increase in lipid peroxidation was observed in the brain of juvenile largemouth bass upon exposure to fullerenes [286]. Administration of Ag, Cu, or Al/Al_2O_3 nanoparticles showed disrupted BBB function and induced brain edema formation [301]. Moreover, silver NPs induced BBB destruction, astrocyte swelling, and neuronal degeneration was observed. Intraperitoneal administration of Ag NPs in mice resulted in the significant alteration in the oxidative stress and antioxidant defense genes in brain suggesting the potential to cause neurotoxicity [305]. NPs once they enter may not be easily eliminated from the brain, could accumulate within the brain to elicit toxic response. However, the self-regenerative ability of neurons is limited. All these findings suggest more cautions needed in biomedical applications of NPs containing products [306]. Therefore, the neurotoxicity of NPs should be carefully evaluated.

23.11 Mechanisms of Nanoparticle Toxicity

The CNTs may exert toxicity by either physical damage to the cells or by activation of signaling mechanisms intracellularly. The physical damage to cell may arise. Insertion of CNT in lipid bilayer resulted in alterations in lipid bilayer organization and leads to increased diffusion of low atomic weight ions and small molecules [307]. Simulation studies suggest that insertion of CNTs in plasma membrane results in formation of pores through which solvated ions can pass [308]. Experimental studies using liposomes and RAW 264.7 cells also confirmed membrane damaging potential of MWCNTs [309]. The disruption of plasma membrane has also been documented in electron microscopy. The MWCNTs may bind to membrane receptor MARCO and disrupt plasma membrane [310, 311].

The role of oxidative stress has been highlighted in several studies [312]. Although it is generally believed that oxidative stress generated due to CNT exposure may result in cell death, the enhanced production of free radicals may not necessarily lead to cell death [313]. Further, treatment with antioxidants failed to abrogate toxicity of MWCNTs in J774.1 cells [310]. Contrary to the in vitro and in vivo studies, MWCNTs do not generate free radicals but may act as free radical scavengers [314]. Oxidative stress may lead to activation of other pathways and DNA damage which may drive the cell machinery to apoptosis [315–317]. Apoptosis is an important mode of cell death in response to CNT exposure. One of the important reasons for in vitro reduction in cell viability and in vivo toxicity appears to be apoptosis. Poly (ADP) ribose polymerase (PARP) activation was observed in response to MWCNTs [316]. The SWCNTs did not induced apoptosis after intravenous administration in mice [169] or in A549 cells [318]. No change in caspase 3 and PARP activity was observed in J774.1 cells incubated with MWCNTs [310]. The role of necrosis in cell death has also been implicated [319]. Cell cycle arrest has also been demonstrated after incubation with SWCNTs [149].

The CNTs may also result in activation of several stress pathways. The activation of transcription factors like NF-kB and AP-1 lead to production of inflammatory cytokines [318]. Incubation of SWCNTs and MWCNTs with mesothelial cells showed an increase in histone 2AX phosphorylation and activation of ERK, JNK, and p38 [316, 320]. Further, AP-1, NF-kB, and Akt activation was also observed after incubation of cells with SWCNTs [150, 316, 321]. The activation of NF-kB and ROS production may lead to production of cytokines [92]. The activation of protein kinases may also lead to activation of NF-kB and could account for toxicity of CNTs. However, MAP kinase activation was not observed in J774.1 cells incubated with MWCNTs [310]. Induction of several other stress related genes following incubation with CNTs has also been demonstrated [315, 322]. Further, CNTs may also lead to altered expression of cytoskeletal and cell adhesion proteins [36, 149, 323].

The role of inflammation in tissue damage has also been observed. Inflammation may arise either due to oxidative stress or physical injury induced by CNTs. Alternatively, the activation of intracellular signaling proteins like NF-kB,

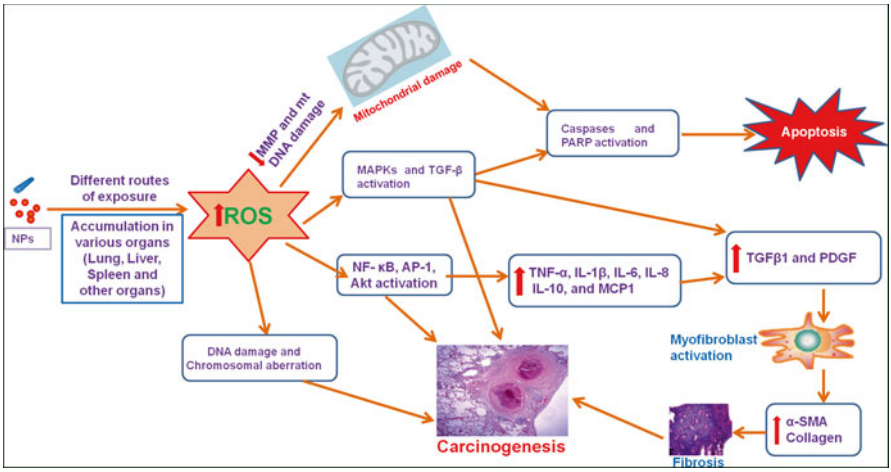

Fig. 23.2 Multitwinning mechanisms—toxic effects of Nanoparticles: Nanoparticles enter the body by various routes and they mainly accumulate in lung, liver, spleen, and other organs depending on the route of entry. These accumulated nanoparticles mainly through reactive oxygen species generation produce toxicity through different intertwining mechanisms. ROS, (1) through mitochondrial membrane potential decrease and mitochondrial DNA damage and (2) through MAPK and TGF-β activation, may activate the caspases pathway which in turn will activate the PARP activation ultimately results in Apoptosis. ROS through NF-κB, AP-1, and Akt activation may promote the over expression of various inflammatory cytokines. These proinflammatory cytokines may increase the activation/overexpression of TGF-β and PGDF, which will result in myofibroblast activation. The activated myofibroblasts produce increased alpha smooth muscle actin and collagen expression, which will result in fibrosis in NPs accumulated organs. Consistent and increased production of collagen together with other mechanisms may sometimes results in carcinogenesis. On the other hand, ROS through (1) various genotoxic effects, (2) through MAPKs and TGF-β activation, and (3) through NF-κB, AP-1, and Akt activation may increase the carcinogenicity potential. (*Abbreviations*: *ROS* reactive oxygen species, *MMP* mitochondrial membrane potential, *PARP* poly ADP ribose polymerase, *MAPKs* mitogen activated protein kinases, *mt DNA* mitochondrial DNA, *TGF-β* transforming growth factor beta, *NK-κB* nuclear factor kappa B, *AP-1* activator protein 1, *Akt* protein kinase B, *TNF-α* tumor necrosis factor alpha, *IL* interleukins, *MCP 1* monocyte chemoattractant protein-1, *PGDF* platelet derived growth factor, *α-SMA* alpha smooth muscle actin)

peroxisome proliferator-activated receptor as well as extracellular signaling molecules like cytokines, PDGF, and TGF could stimulate downstream signaling pathways resulting in oxidative stress, inflammatory response, chemotaxis, fibrosis, and cell death at the site of CNT administration [324–341]. Additionally, CNTs may also induce inflammasome activation [342–347]. Further, CNTs may also stimulate apoptotic cell death pathways involving mitochondria (Fig. 23.2) [348–350].

The CNTs may bind to several proteins in plasma and cells including complement proteins and lung surfactant proteins [351–353]. Further, CNTs may also lead to activation of the complement system [351, 352].

23.12 Nanotoxicology: Regulatory Prospective

Despite increasing population exposure to different nanoparticles, their potential genotoxicity remains controversial. The implementation of well-accepted toxicity assays for testing nanomaterials remains a controversial issue. This is because many of these toxicity tests were designed for screening general macroparticle chemicals and might not be suitable for the screening of nanomaterials (even of the same compositional material). Furthermore, no nanoparticle-type positive controls have been established or universally accepted for these tests. Although several genetic toxicology tests have been validated for chemicals according to the Organisation for Economic Co-operation and Development (OECD) test guidelines, the relevance of these assays for nanoparticulate materials remains to be determined. In an attempt to remedy this issue, the OECD has established current projects designed to evaluate the relevance and reproducibility of safety hazard tests for representative nanomaterials, including genotoxicity assays (i.e., Steering Group 3—Safety Testing of Representative Nanomaterials). It seems clear that the development of standardized approaches will be necessary in order to determine whether exposures to specific nanoparticle-types are associated with toxic events [354].

23.13 Challenges in NPs Toxicity Screening

23.13.1 Interference with Cell Viability Assays

The NPs have been demonstrated to interfere with cell viability assays [13, 355–357]. Neutral red assay is based on the principle that live cells with intact lysosomes accumulate the dye. The extent of dye uptake is directly related to the number of viable cells. Carbon-based NPs can adsorb the dye and show lack of toxicity [358]. An increase in lamellar bodies may also be responsible for higher accumulation of the dye inside cells [359]. Similarly, higher viability was observed with MTT assay while no significant artifacts were observed with WST-1 dye. The MTT formazon crystals were found to be attached to SWCNTs while WST-1 formazon did not attached. Further, no cytotoxicity was observed in LDH leakage or mitochondrial membrane potential assay [357]. NPs can interference in other cell viability assays as well, viz., Alamar Blue, Coomassie Blue, Trypan Blue, Calcein-AM [13, 356]. The interference with MTT assay may also depend on the type of surfactant used to suspend the NPs [318, 355]. Spectroscopic studies also demonstrated interaction of several dyes with the commonly used reagents in cell viability assays [356].

23.13.2 Adsorption of Biomolecules

Due to their inherent adsorptive properties, carbon-based NPs may also adsorb several biochemicals markers leading to misleading results. Carbon black has been reported to adsorb IL-8 leading to reduction in estimated amounts of the cytokine [358]. Similar effects of CNTs may also be expected for CNTs as well. Similarly, CNTs adsorb several proteins in vitro and in vivo [351–353, 360, 361]. The endogenous proteins may bind to the CNTs leading to displacement of CNT stabilizing surfactant molecules [362]. The CNTs have been shown to adsorb several aromatic compounds [363–366]. Due to this property, CNTs may adsorb several chemical constituents of the culture medium [367]. This may lead to depletion of the culture medium leading to false toxicity [368, 369]. Further, CNTs may also bind to biomolecules which may further influence their toxicity profile [370–374].

23.13.3 Role of Impurities

The CNT synthesis is catalyzed in presence of metals like iron (Fe) and nickel (Ni). These metals remain entrapped in the raw CNT preparations but can be removed by refluxing the CNTs in acid. Use of raw CNTs containing high metal content can lead to leeching of the metals from CNTs which may lead to cell death [375, 376]. However, contradictory reports on the role of residual metal content have appeared. The presence of iron can lead to production of higher amounts of free radicals as compared to iron-stripped CNTs in RAW 264.7 cells [377]. Similarly, metal impurity-dependent cytotoxicity was demonstrated in other cells also [313, 378]. Presence of iron in MWCNTs has been demonstrated to affect neuronal differentiation in PC12 cells and catecholamine release in chromaffin cells [379, 380]. Purified SWCNTs were found to less toxic in several cell types as compared to unpurified to SWCNTs [381]. On the other hand, presence of metal impurities had no effect on A549 cell viability [382].

23.13.4 NPs Length

CNTs are available in various lengths ranging from to a few hundred nanometers to hundreds of micrometers. The CNT bulk material is also not a monodisperse system but consist of a mixture of CNTs with varying lengths. The efficiency of cellular uptake may be dependent of CNT length [383]. Further, longer tubes may entangle with other tubes and aggregate. Thus, CNT length may play a critical role in dictating the toxicity of CNTs. The high aspect ratio of CNTs may impart high toxicity to these materials [83, 384]. The small length MWCNTs were found to better dispersed in rat lungs as compared to longer MWCNTs and showed size-dependent differences in distribution of inflammation sites. In vitro, shorter MWCNTs showed

concentration-dependent cytotoxicity and TNF-α production. The size-dependent differences in in vitro and in vivo experiments were attributed to higher degree of aggregation in longer CNTs [161]. Subcutaneous implantation of CNTs showed differences in distribution of CNTs at the injection site. While shorter CNTs were present inside macrophages, longer CNTs could be observed in macrophages and intercellular spaces. The inflammation appeared to be more pronounced with longer CNTs. In vitro, induction of THP-1 cells appeared to be independent of CNT length [385, 386]. Similarly, the in vitro toxicity of MWCNTs appeared to be independent of size in A549 cells [382, 387].

23.13.5 NPs Aggregation

The aggregation of NPs leads to inhomogeneity in the NPs suspension. This leads to variability in NPs concentration/dose between different samples drawn from the same suspension. In vitro toxicity studies showed that aggregation of CNTs leads to floating aggregates which do not come in contact with the cells and show false negative toxicity results [161]. The rope-like aggregates were found to be more toxic as compared to dispersed CNTs [388]. Further, well dispersed CNTs may be less toxic in vivo [389].

23.13.6 NPs Surface Chemistry

The surface chemistry of NPs may play an important role in determining the toxicity and fate of NPs. PEG-functionalized SWCNTs show less toxicity as compared to pristine CNTs and remain the cytoplasm. Similarly, PEI-wrapped SWCNTs are less toxic than pristine SWCNTs but localize in vacuoles and nucleus [390]. Similarly, cells incubated with glycodendrimer-coated SWCNTs showed no change in cell viability [391]. The type of functionalization can also reduce plasma protein adsorption and degree of complement activation by MWCNTs [352]. The adsorption of plasma or albumin on SWCNTs may lead to a change in hydrophobicity which can alter interaction of CNTs with cells [381]. Adsorption of antibodies on SWCNTs can also increase T-cell activation [392]. In vivo, functionalization can also alter the biodistribution and pharmacokinetics of CNTs [38, 393].

23.13.7 Method/Route of Administration

The method and route of administration of CNTs also determine the degree of toxicity. In general, inhalation toxicity of NPs is significantly higher as compared to intravenous route. This is because inflammation is more pronounced in lungs as compared to systemic administration.

23.14 Knowledge Gaps in Nanoparticle Toxicity Evaluation

The available literature showing presence/absence of NPs toxicity is fragmentary. The interpretation of data is further compounded by incomplete or lack of NPs characterization. The study design is variable between different research groups with variations in time points, NPs concentration, surfactant type and concentration, NPs suppliers, nature of impurities, positive and negative controls, end points, etc. It becomes imperative to harmonize the NPs studies in order to achieve suitability of NPs toxicity data for arriving at a meaningful conclusion. There is a need for proper physical and chemical characterization of NPs which includes, but is not limited to, the nature and quantity of NPs, diameter, size, and length distribution, nature and quantity of impurities, and degree of surface functionalization.

The pristine CNTs are long and contain several types of impurities. The CNTs are acid-treated to remove impurities and "cut" CNTs to the desired length. This acid treatment results in functionalization of the CNT surface with hydroxyl and carboxylic groups. The aqueous dispersibility and toxicity is markedly affected by the degree of functionalization. This can be characterized by Raman spectroscopy and infrared spectroscopy. The CNTs are hydrophobic in nature and require a suspending agent to stabilize them. Several kinds of suspending agents are reported in literature which produce CNT suspensions with varying degrees of agglomeration and surface charge. Further, these suspending agents, particularly surfactants, may themselves be toxic and produce false positive toxicity data. Additionally, these surfactants may, by themselves, be nontoxic but may sensitize the cells to CNT toxicity. Further, the surfactant coating on CNT may prevent direct contact of CNT with cells and may result in false negative toxicity result. Thus, it is required that CNT samples be characterized for degree of agglomeration prior to experimentation. This may be achieved by dynamic laser scattering or UV–visible spectroscopy. The use of electron microscopy and atomic force microscopy may also provide useful information. It is necessary to harmonize the use of surfactants to achieve uniformity in toxicity data. The use of surfactant alone is not sufficient to suspend CNTs in aqueous media and require sonication of CNT samples to achieve dispersion. The sonication procedures may lead to reduction in CNT length and generate functional groups on the CNT surface. The changes in size may be determined by electron and atomic force microscopy while the chemical changes may be determined by infrared and nuclear magnetic resonance spectroscopy.

The toxicity of NPs is dose/concentration dependent. The in vivo doses vary significantly among researchers. Similarly, the highest concentrations of NPs used in in vitro studies vary greatly. It may be argued that the use of too high concentrations of NPs may lead to nonspecific toxic effects which may arise due to media depletion or changes in osmolarity and pH of the medium. It becomes necessary to set the highest concentration of NPs to be tested and the generally acceptable endpoints to be determined. Such nonspecific effects may also appear in vivo due to differences in osmolarity and pH of the injected material. Such differences may lead to an inflammatory response as well. The heterogeneity in time points also

leads to misleading results. In vitro, short durations of incubation may lead to false negative toxicity data. Similarly, in vivo, toxicity may not be observed after short durations following injection. However, if the toxicity is reversible, the toxic effects may be reverted after prolonged durations. Hence, suitable time points need to be determined.

23.15 Future Trends

The current strategies for determining the toxicity of NPs are not sufficient. This is because of difficulties in quantification of NPs in biological samples and interference in estimation of toxicity. There is a need to establish toxicity standards which includes identification of suitable positive and negative controls. The commonly used negative control, carbon black, may not be suitable [358].

Newer, noninvasive techniques need to be employed to determine the in vivo disposition and fate of NPs over long durations [394–396]. Similarly, newer imaging techniques need to be applied to determine the dynamics and quantify the in vitro uptake of NPs [397–399]. High content and single cell imaging may be employed to determine the cellular effects of NPs. Alternate assay methodologies need to be investigated to determine the cytotoxic potential of NPs [400]. The application of genomics technologies can shed useful information on the mechanism of toxicity [91].

The use of combinatorial chemistry along with the application of high-throughput biological assay procedures can help develop biocompatible NPs. This, in conjunction with *in silico* designing, may be a useful tool to identify NPs with desired physicochemical characteristics and biocompatibility [401].

References

1. Martin RS, Mather TA, Pyle DM, Power M, Allen AG, Aiuppa A, Horwell CJ, Ward EPW (2008) Composition-resolved size distributions of volcanic aerosols in the Mt. Etna plumes. J Geophys Res 113, D17211:1–17
2. Murr LE, Soto KF, Garza KM, Guerrero PA, Martinez F, Esquivel EV, Ramirez DA, Shi Y, Bang JJ, Venzor J 3rd (2006) Combustion-generated nanoparticulates in the El Paso, TX, USA / Juarez, Mexico Metroplex: their comparative characterization and potential for adverse health effects. Int J Environ Res Public Health 3:48–66
3. Murr LE (2007) Nanoparticulate materials in antiquity: the good, the bad and the ugly. Microsc Microanal 13:1118–1119
4. Farokhzad OC, Langer R (2009) Impact of nanotechnology on drug delivery. ACS Nano 3:16–20
5. Hong H, Gao T, Cai W (2009) Molecular imaging with single-walled carbon nanotubes. Nano Today 4:252–261
6. Sandhiya S, Dkhar SA, Surendiran A (2009) Emerging trends of nanomedicine: an overview. Fundam Clin Pharmacol 23:263–269

7. Moghimi SM, Hunter AC, Murray JC (2005) Nanomedicine: current status and future prospects. FASEB J 19:311–330
8. Yu X, Zhang Y, Chen C, Yao Q, Li M (2010) Targeted drug delivery in pancreatic cancer. Biochim Biophys Acta 1805:97–104
9. Tang MF, Lei L, Guo SR, Huang WL (2010) Recent progress in nanotechnology for cancer therapy. Chin J Cancer 29:775–780
10. Rao J (2008) Shedding light on tumors using nanoparticles. ACS Nano 2:1984–1986
11. Inoue K-i, Takano H, Yanagisawa R, Koike E, Shimada A (2009) Size effects of latex nanomaterials on lung inflammation in mice. Toxicol Appl Pharmacol 234:68–76
12. Aillon KL, Xie Y, El-Gendy N, Berkland CJ, Forrest ML (2009) Effects of nanomaterial physicochemical properties on in vivo toxicity. Adv Drug Deliv Rev 61:457–466
13. Monteiro-Riviere NA, Inman AO, Zhang LW (2009) Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol Appl Pharmacol 234:222–235
14. Choi JY, Ramachandran G, Kandlikar M (2009) The impact of toxicity testing costs on nanomaterial regulation. Environ Sci Technol 43:3030–3034
15. Lee HM, Shin DM, Song HM, Yuk JM, Lee ZW, Lee SH, Hwang SM, Kim JM, Lee CS, Jo EK (2009) Nanoparticles up-regulate tumor necrosis factor-alpha and CXCL8 via reactive oxygen species and mitogen-activated protein kinase activation. Toxicol Appl Pharmacol 238:160–169
16. Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, Radomski MW (2005) Nanoparticle-induced platelet aggregation and vascular thrombosis. Br J Pharmacol 146:882–893
17. Singh N, Manshian B, Jenkins GJ, Griffiths SM, Williams PM, Maffeis TG, Wright CJ, Doak SH (2009) NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30:3891–3914
18. Murray AR, Kisin E, Leonard SS, Young SH, Kommineni C, Kagan VE, Castranova V, Shvedova AA (2009) Oxidative stress and inflammatory response in dermal toxicity of single-walled carbon nanotubes. Toxicology 257:161–171
19. Colvin VL (2003) The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21:1166–1170
20. Shvedova AA, Kagan VE, Fadeel B (2010) Close encounters of the small kind: adverse effects of man-made materials interfacing with the nano-cosmos of biological systems. Annu Rev Pharmacol Toxicol 50:63–88
21. Stevens MM (2009) Toxicology: testing in the third dimension. Nat Nanotechnol 4:342–343
22. Prato M, Kostarelos K, Bianco A (2008) Functionalized carbon nanotubes in drug design and discovery. Acc Chem Res 41:60–68
23. Kam NW, O'Connell M, Wisdom JA, Dai H (2005) Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. Proc Natl Acad Sci U S A 102:11600–11605
24. Kostarelos K, Bianco A, Prato M (2009) Promises, facts and challenges for carbon nanotubes in imaging and therapeutics. Nat Nanotechnol 4:627–633
25. Liu Z, Chen K, Davis C, Sherlock S, Cao Q, Chen X, Dai H (2008) Drug delivery with carbon nanotubes for in vivo cancer treatment. Cancer Res 68:6652–6660
26. Zhang Y, Bai Y, Yan B (2010) Functionalized carbon nanotubes for potential medicinal applications. Drug Discov Today 15:428–435
27. Bianco A (2004) Carbon nanotubes for the delivery of therapeutic molecules. Expert Opin Drug Deliv 1:57–65
28. Lacerda L, Bianco A, Prato M, Kostarelos K (2006) Carbon nanotubes as nanomedicines: from toxicology to pharmacology. Adv Drug Deliv Rev 58:1460–1470
29. Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand JP, Prato M, Kostarelos K, Bianco A (2004) Functionalized carbon nanotubes for plasmid DNA gene delivery. Angew Chem Int Ed Engl 43:5242–5246

30. Pastorin G (2009) Crucial functionalizations of carbon nanotubes for improved drug delivery: a valuable option? Pharm Res 26:746–769
31. Fonseca C, Simoes S, Gaspar R (2002) Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. J Control Release 83:273–286
32. Lo CT, Van Tassel PR, Saltzman WM (2010) Poly(lactide-co-glycolide) nanoparticle assembly for highly efficient delivery of potent therapeutic agents from medical devices. Biomaterials 31:3631–3642
33. Wang ZH, Wang ZY, Sun CS, Wang CY, Jiang TY, Wang SL (2010) Trimethylated chitosan-conjugated PLGA nanoparticles for the delivery of drugs to the brain. Biomaterials 31:908–915
34. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, Bawendi MG, Frangioni JV (2007) Renal clearance of quantum dots. Nat Biotechnol 25:1165–1170
35. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307:538–544
36. Erdely A, Hulderman T, Salmen R, Liston A, Zeidler-Erdely PC, Schwegler-Berry D, Castranova V, Koyama S, Kim YA, Endo M, Simeonova PP (2009) Cross-talk between lung and systemic circulation during carbon nanotube respiratory exposure. Potential biomarkers. Nano Lett 9:36–43
37. Ji Z, Zhang D, Li L, Shen X, Deng X, Dong L, Wu M, Liu Y (2009) The hepatotoxicity of multi-walled carbon nanotubes in mice. Nanotechnology 20:445101
38. Lacerda L, Ali-Boucetta H, Herrero MA, Pastorin G, Bianco A, Prato M, Kostarelos K (2008) Tissue histology and physiology following intravenous administration of different types of functionalized multiwalled carbon nanotubes. Nanomedicine (Lond) 3:149–161
39. Muller J, Delos M, Panin N, Rabolli V, Huaux F, Lison D (2009) Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat. Toxicol Sci 110:442–448
40. Schipper ML, Nakayama-Ratchford N, Davis CR, Kam NW, Chu P, Liu Z, Sun X, Dai H, Gambhir SS (2008) A pilot toxicology study of single-walled carbon nanotubes in a small sample of mice. Nat Nanotechnol 3:216–221
41. Singh R, Pantarotto D, Lacerda L, Pastorin G, Klumpp C, Prato M, Bianco A, Kostarelos K (2006) Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers. Proc Natl Acad Sci U S A 103:3357–3362
42. Chan VS (2006) Nanomedicine: an unresolved regulatory issue. Regul Toxicol Pharmacol 46:218–224
43. Kipen HM, Laskin DL (2005) Smaller is not always better: nanotechnology yields nanotoxicology. Am J Physiol Lung Cell Mol Physiol 289:L696–L697
44. Oberdorster G, Oberdorster E, Oberdorster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839
45. Selvi BR, Jagadeesan D, Suma BS, Nagashankar G, Arif M, Balasubramanyam K, Eswaramoorthy M, Kundu TK (2008) Intrinsically fluorescent carbon nanospheres as a nuclear targeting vector: delivery of membrane-impermeable molecule to modulate gene expression in vivo. Nano Lett 8:3182–3188
46. Levi-Polyachenko NH, Merkel EJ, Jones BT, Carroll DL, Stewart JH (2009) Rapid photothermal intracellular drug delivery using multiwalled carbon nanotubes. Mol Pharm 6:1092–1099
47. Ma L, Liu J, Li N, Wang J, Duan Y, Yan J, Liu H, Wang H, Hong F (2010) Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO2 delivered to the abdominal cavity. Biomaterials 31:99–105
48. Belyanskaya L, Weigel S, Hirsch C, Tobler U, Krug HF, Wick P (2009) Effects of carbon nanotubes on primary neurons and glial cells. Neurotoxicology 30:702–711
49. Hu YL, Gao JQ (2010) Potential neurotoxicity of nanoparticles. Int J Pharm 394:115–121

50. Jones R (2009) It's not just about nanotoxicology. Nat Nanotechnol 4:615
51. Kim S, Choi JE, Choi J, Chung KH, Park K, Yi J, Ryu DY (2009) Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. Toxicol In Vitro 23:1076–1084
52. Mayer A, Vadon M, Rinner B, Novak A, Wintersteiger R, Frohlich E (2009) The role of nanoparticle size in hemocompatibility. Toxicology 258:139–147
53. Napierska D, Thomassen LC, Rabolli V, Lison D, Gonzalez L, Kirsch-Volders M, Martens JA, Hoet PH (2009) Size-dependent cytotoxicity of monodisperse silica nanoparticles in human endothelial cells. Small 5:846–853
54. Valant J, Drobne D, Sepcic K, Jemec A, Kogej K, Kostanjsek R (2009) Hazardous potential of manufactured nanoparticles identified by in vivo assay. J Hazard Mater 171:160–165
55. Fischer HC, Chan WC (2007) Nanotoxicity: the growing need for in vivo study. Curr Opin Biotechnol 18:565–571
56. Fadeel B, Valerian H, Krug H, Shvedova A, Svartengren M, Tran L, Wiklund L (2007) There's plenty of room at the forum: potential risks and safety assessment of engineered nanomaterials. Nanotoxicology 1:73–84
57. Ray PC, Yu H, Fu PP (2009) Toxicity and environmental risks of nanomaterials: challenges and future needs. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27:1–35
58. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ (2004) Nanotoxicology. Occup Environ Med 61:727–728
59. Haynes CL (2010) The emerging field of nanotoxicology. Anal Bioanal Chem 398:587–588
60. Service RF (2008) Science policy. Report faults U.S. strategy for nanotoxicology research. Science 322:1779
61. Kagan VE, Bayir H, Shvedova AA (2005) Nanomedicine and nanotoxicology: two sides of the same coin. Nanomedicine 1:313–316
62. Rzigalinski BA, Strobl JS (2009) Cadmium-containing nanoparticles: perspectives on pharmacology and toxicology of quantum dots. Toxicol Appl Pharmacol 238:280–288
63. Kunzmann A, Andersson B, Thurnherr T, Krug H, Scheynius A, Fadeel B (2011) Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation. Biochim Biophys Acta 1810(3):361–373
64. Suh WH, Suslick KS, Stucky GD, Suh YH (2009) Nanotechnology, nanotoxicology, and neuroscience. Prog Neurobiol 87:133–170
65. Kroll A, Pillukat MH, Hahn D, Schnekenburger J (2009) Current in vitro methods in nanoparticle risk assessment: limitations and challenges. Eur J Pharm Biopharm 72:370–377
66. Kahru A, Savolainen K (2010) Potential hazard of nanoparticles: from properties to biological and environmental effects. Toxicology 269:89–91
67. Casalsa E, Vázquez-Camposa S, Bastús NG, Puntes V (2008) Distribution and potential toxicity of engineered inorganic nanoparticles and carbon nanostructures in biological systems. Trends Anal Chem 27:672–683
68. Jones CF, Grainger DW (2009) In vitro assessments of nanomaterial toxicity. Adv Drug Deliv Rev 61:438–456
69. Geys J, Nemery B, Hoet PH (2010) Assay conditions can influence the outcome of cytotoxicity tests of nanomaterials: better assay characterization is needed to compare studies. Toxicol In Vitro 24:620–629
70. Clift MJ, Gehr P, Rothen-Rutishauser B (2011) Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative. Arch Toxicol 85:723–731
71. Patra CR, Abdel Moneim SS, Wang E, Dutta S, Patra S, Eshed M, Mukherjee P, Gedanken A, Shah VH, Mukhopadhyay D (2009) In vivo toxicity studies of europium hydroxide nanorods in mice. Toxicol Appl Pharmacol 240:88–98
72. Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K (2009) Silica nanoparticles as hepatotoxicants. Eur J Pharm Biopharm 72:496–501
73. Aggarwal P, Hall JB, McLeland CB, Dobrovolskaia MA, McNeil SE (2009) Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. Adv Drug Deliv Rev 61:428–437

74. Li SD, Huang L (2008) Pharmacokinetics and biodistribution of nanoparticles. Mol Pharm 5:496–504
75. Muller J, Huaux F, Lison D (2006) Respiratory toxicity of carbon nanotubes: how worried should we be? Carbon 44:1048–1056
76. Lam CW, James JT, McCluskey R, Hunter RL (2004) Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol Sci 77:126–134
77. Chou CC, Hsiao HY, Hong QS, Chen CH, Peng YW, Chen HW, Yang PC (2008) Single-walled carbon nanotubes can induce pulmonary injury in mouse model. Nano Lett 8:437–445
78. Liu Z, Davis C, Cai W, He L, Chen X, Dai H (2008) Circulation and long-term fate of functionalized, biocompatible single-walled carbon nanotubes in mice probed by Raman spectroscopy. Proc Natl Acad Sci U S A 105:1410–1415
79. Fadeel B, Garcia-Bennett AE (2010) Better safe than sorry: understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. Adv Drug Deliv Rev 62:362–374
80. Mutlu GM, Budinger GR, Green AA, Urich D, Soberanes S, Chiarella SE, Alheid GF, McCrimmon DR, Szleifer I, Hersam MC (2010) Biocompatible nanoscale dispersion of single-walled carbon nanotubes minimizes in vivo pulmonary toxicity. Nano Lett 10:1664–1670
81. Vittorio O, Raffa V, Cuschieri A (2009) Influence of purity and surface oxidation on cytotoxicity of multiwalled carbon nanotubes with human neuroblastoma cells. Nanomedicine 5:424–431
82. Ryman-Rasmussen JP, Riviere JE, Monteiro-Riviere NA (2007) Surface coatings determine cytotoxicity and irritation potential of quantum dot nanoparticles in epidermal keratinocytes. J Invest Dermatol 127:143–153
83. Grabinski C, Hussain S, Lafdi K, Braydich-Stolle L, Schlager J (2007) Effect of particle dimension on biocompatibility of carbon nanomaterials. Carbon 45:2828–2835
84. Reddy AR, Reddy YN, Krishna DR, Himabindu V (2010) Multi wall carbon nanotubes induce oxidative stress and cytotoxicity in human embryonic kidney (HEK293) cells. Toxicology 272:11–16
85. Carlson C, Hussain SM, Schrand AM, Braydich-Stolle LK, Hess KL, Jones RL, Schlager JJ (2008) Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. J Phys Chem B 112:13608–13619
86. Fubini B, Ghiazza M, Fenoglio I (2010) Physico-chemical features of engineered nanoparticles relevant to their toxicity. Nanotoxicology 4:347–363
87. Ahamed M, Posgai R, Gorey TJ, Nielsen M, Hussain SM, Rowe JJ (2010) Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in Drosophila melanogaster. Toxicol Appl Pharmacol 242:263–269
88. Elgrabli D, Abella-Gallart S, Robidel F, Rogerieux F, Boczkowski J, Lacroix G (2008) Induction of apoptosis and absence of inflammation in rat lung after intratracheal instillation of multiwalled carbon nanotubes. Toxicology 253:131–136
89. Kang SJ, Kim BM, Lee YJ, Chung HW (2008) Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. Environ Mol Mutagen 49:399–405
90. Moller P, Jacobsen NR, Folkmann JK, Danielsen PH, Mikkelsen L, Hemmingsen JG, Vesterdal LK, Forchhammer L, Wallin H, Loft S (2010) Role of oxidative damage in toxicity of particulates. Free Radic Res 44:1–46
91. Poma A, Di Giorgio ML (2008) Toxicogenomics to improve comprehension of the mechanisms underlying responses of in vitro and in vivo systems to nanomaterials: a review. Curr Genomics 9:571–585
92. Ye SF, Wu YH, Hou ZQ, Zhang QQ (2009) ROS and NF-kappaB are involved in upregulation of IL-8 in A549 cells exposed to multi-walled carbon nanotubes. Biochem Biophys Res Commun 379:643–648

93. Park EJ, Kim H, Kim Y, Yi J, Choi K, Park K (2010) Carbon fullerenes (C60s) can induce inflammatory responses in the lung of mice. Toxicol Appl Pharmacol 244:226–233
94. Park YH, Kim JN, Jeong SH, Choi JE, Lee SH, Choi BH, Lee JP, Sohn KH, Park KL, Kim MK, Son SW (2010) Assessment of dermal toxicity of nanosilica using cultured keratinocytes, a human skin equivalent model and an in vivo model. Toxicology 267:178–181
95. De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJ, Geertsma RE (2008) Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29:1912–1919
96. Balasubramanian SK, Jittiwat J, Manikandan J, Ong CN, Yu LE, Ong WY (2010) Biodistribution of gold nanoparticles and gene expression changes in the liver and spleen after intravenous administration in rats. Biomaterials 31:2034–2042
97. Cho WS, Cho M, Jeong J, Choi M, Han BS, Shin HS, Hong J, Chung BH, Jeong J, Cho MH (2010) Size-dependent tissue kinetics of PEG-coated gold nanoparticles. Toxicol Appl Pharmacol 245:116–123
98. Li JJ, Hartono D, Ong CN, Bay BH, Yung LY (2010) Autophagy and oxidative stress associated with gold nanoparticles. Biomaterials 31:5996–6003
99. Ahamed M, Karns M, Goodson M, Rowe J, Hussain SM, Schlager JJ, Hong Y (2008) DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. Toxicol Appl Pharmacol 233:404–410
100. Chu M, Wu Q, Yang H, Yuan R, Hou S, Yang Y, Zou Y, Xu S, Xu K, Ji A, Sheng L (2010) Transfer of quantum dots from pregnant mice to pups across the placental barrier. Small 6:670–678
101. Rosas-Hernandez H, Jimenez-Badillo S, Martinez-Cuevas PP, Gracia-Espino E, Terrones H, Terrones M, Hussain SM, Ali SF, Gonzalez C (2009) Effects of 45-nm silver nanoparticles on coronary endothelial cells and isolated rat aortic rings. Toxicol Lett 191:305–313
102. Mo Y, Wan R, Chien S, Tollerud DJ, Zhang Q (2009) Activation of endothelial cells after exposure to ambient ultrafine particles: the role of NADPH oxidase. Toxicol Appl Pharmacol 236:183–193
103. Ema M, Kobayashi N, Naya M, Hanai S, Nakanishi J (2010) Reproductive and developmental toxicity studies of manufactured nanomaterials. Reprod Toxicol 30:343–352
104. Gao J, Wang HL, Shreve A, Iyer R (2010) Fullerene derivatives induce premature senescence: a new toxicity paradigm or novel biomedical applications. Toxicol Appl Pharmacol 244:130–143
105. Johnston HJ, Hutchison GR, Christensen FM, Aschberger K, Stone V (2010) The biological mechanisms and physicochemical characteristics responsible for driving fullerene toxicity. Toxicol Sci 114:162–182
106. Naha PC, Bhattacharya K, Tenuta T, Dawson KA, Lynch I, Gracia A, Lyng FM, Byrne HJ (2010) Intracellular localisation, geno- and cytotoxic response of polyN-isopropylacrylamide (PNIPAM) nanoparticles to human keratinocyte (HaCaT) and colon cells (SW 480). Toxicol Lett 198:134–143
107. Marquis BJ, Love SA, Braun KL, Haynes CL (2009) Analytical methods to assess nanoparticle toxicity. Analyst 134:425–439
108. Vega-Villa KR, Takemoto JK, Yanez JA, Remsberg CM, Forrest ML, Davies NM (2008) Clinical toxicities of nanocarrier systems. Adv Drug Deliv Rev 60:929–938
109. Lei R, Wu C, Yang B, Ma H, Shi C, Wang Q, Wang Q, Yuan Y, Liao M (2008) Integrated metabolomic analysis of the nano-sized copper particle-induced hepatotoxicity and nephrotoxicity in rats: a rapid in vivo screening method for nanotoxicity. Toxicol Appl Pharmacol 232:292–301
110. Bu Q, Yan G, Deng P, Peng F, Lin H, Xu Y, Cao Z, Zhou T, Xue A, Wang Y, Cen X, Zhao YL (2010) NMR-based metabonomic study of the sub-acute toxicity of titanium dioxide nanoparticles in rats after oral administration. Nanotechnology 21:125105
111. Lubick N (2008) Risks of nanotechnology remain uncertain. Environ Sci Technol 42:1821–1824

112. Seaton A, Tran L, Aitken R, Donaldson K (2010) Nanoparticles, human health hazard and regulation. J R Soc Interface 7(Suppl 1):S119–S129
113. Stone V, Donaldson K (2006) Nanotoxicology: signs of stress. Nat Nanotechnol 1:23–24
114. George S, Pokhrel S, Xia T, Gilbert B, Ji Z, Schowalter M, Rosenauer A, Damoiseaux R, Bradley KA, Madler L, Nel AE (2010) Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping. ACS Nano 4:15–29
115. Meng H, Xia T, George S, Nel AE (2009) A predictive toxicological paradigm for the safety assessment of nanomaterials. ACS Nano 3:1620–1627
116. Foldvari M, Bagonluri M (2008) Carbon nanotubes as functional excipients for nanomedicines: II. Drug delivery and biocompatibility issues. Nanomedicine 4:183–200
117. Chikkaveeraiah BV, Bhirde A, Malhotra R, Patel V, Gutkind JS, Rusling JF (2009) Single-wall carbon nanotube forest arrays for immunoelectrochemical measurement of four protein biomarkers for prostate cancer. Anal Chem 81:9129–9134
118. Liu Z, Tabakman SM, Chen Z, Dai H (2009) Preparation of carbon nanotube bioconjugates for biomedical applications. Nat Protoc 4:1372–1382
119. Bianco A, Kostarelos K, Partidos CD, Prato M (2005) Biomedical applications of functionalised carbon nanotubes. Chem Commun (Camb) 5:571–577
120. Li X, Fan Y, Watari F (2010) Current investigations into carbon nanotubes for biomedical application. Biomed Mater 5:22001
121. Yandar N, Pastorin G, Prato M, Bianco A, Patarroyo ME, Manuel Lozano J (2008) Immunological profile of a Plasmodium vivax AMA-1N-terminus peptide-carbon nanotube conjugate in an infected Plasmodium berghei mouse model. Vaccine 26:5864–5873
122. Klumpp C, Kostarelos K, Prato M, Bianco A (2006) Functionalized carbon nanotubes as emerging nanovectors for the delivery of therapeutics. Biochim Biophys Acta 1758:404–412
123. Krajcik R, Jung A, Hirsch A, Neuhuber W, Zolk O (2008) Functionalization of carbon nanotubes enables non-covalent binding and intracellular delivery of small interfering RNA for efficient knock-down of genes. Biochem Biophys Res Commun 369:595–602
124. Liu Z, Winters M, Holodniy M, Dai H (2007) siRNA delivery into human T cells and primary cells with carbon-nanotube transporters. Angew Chem Int Ed Engl 46:2023–2027
125. Zhang Z, Yang X, Zhang Y, Zeng B, Wang S, Zhu T, Roden RB, Chen Y, Yang R (2006) Delivery of telomerase reverse transcriptase small interfering RNA in complex with positively charged single-walled carbon nanotubes suppresses tumor growth. Clin Cancer Res 12:4933–4939
126. Podesta JE, Al-Jamal KT, Herrero MA, Tian B, Ali-Boucetta H, Hegde V, Bianco A, Prato M, Kostarelos K (2009) Antitumor activity and prolonged survival by carbon-nanotube-mediated therapeutic siRNA silencing in a human lung xenograft model. Small 5:1176–1185
127. Chaudhuri P, Soni S, Sengupta S (2010) Single-walled carbon nanotube-conjugated chemotherapy exhibits increased therapeutic index in melanoma. Nanotechnology 21:025102
128. Liu Z, Cai W, He L, Nakayama N, Chen K, Sun X, Chen X, Dai H (2007) In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. Nat Nanotechnol 2:47–52
129. Teker K (2008) Bioconjugated carbon nanotubes for targeting cancer biomarkers. Mater Sci Eng B 153:83–87
130. Ji SR, Liu C, Zhang B, Yang F, Xu J, Long J, Jin C, Fu DL, Ni QX, Yu XJ (2010) Carbon nanotubes in cancer diagnosis and therapy. Biochim Biophys Acta 1806:29–35
131. Bhirde AA, Patel V, Gavard J, Zhang G, Sousa AA, Masedunskas A, Leapman RD, Weigert R, Gutkind JS, Rusling JF (2009) Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. ACS Nano 3:307–316
132. Zhang X, Meng L, Lu Q, Fei Z, Dyson PJ (2009) Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. Biomaterials 30:6041–6047

133. Wu W, Li R, Bian X, Zhu Z, Ding D, Li X, Jia Z, Jiang X, Hu Y (2009) Covalently combining carbon nanotubes with anticancer agent: preparation and antitumor activity. ACS Nano 3:2740–2750
134. Frangioni JV (2003) In vivo near-infrared fluorescence imaging. Curr Opin Chem Biol 7:626–634
135. Marches R, Chakravarty P, Musselman IH, Bajaj P, Azad RN, Pantano P, Draper RK, Vitetta ES (2009) Specific thermal ablation of tumor cells using single-walled carbon nanotubes targeted by covalently-coupled monoclonal antibodies. Int J Cancer 125:2970–2977
136. Ghosh S, Dutta S, Gomes E, Carroll D, D'Agostino R Jr, Olson J, Guthold M, Gmeiner WH (2009) Increased heating efficiency and selective thermal ablation of malignant tissue with DNA-encased multiwalled carbon nanotubes. ACS Nano 3:2667–2673
137. De la Zerda A, Zavaleta C, Keren S, Vaithilingam S, Bodapati S, Liu Z, Levi J, Smith BR, Ma TJ, Oralkan O, Cheng Z, Chen X, Dai H, Khuri-Yakub BT, Gambhir SS (2008) Carbon nanotubes as photoacoustic molecular imaging agents in living mice. Nat Nanotechnol 3:557–562
138. Burke A, Ding X, Singh R, Kraft RA, Levi-Polyachenko N, Rylander MN, Szot C, Buchanan C, Whitney J, Fisher J, Hatcher HC, D'Agostino R Jr, Kock ND, Ajayan PM, Carroll DL, Akman S, Torti FM, Torti SV (2009) Long-term survival following a single treatment of kidney tumors with multiwalled carbon nanotubes and near-infrared radiation. Proc Natl Acad Sci U S A 106:12897–12902
139. Chakravarty P, Marches R, Zimmerman NS, Swafford AD, Bajaj P, Musselman IH, Pantano P, Draper RK, Vitetta ES (2008) Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes. Proc Natl Acad Sci U S A 105:8697–8702
140. Keren S, Zavaleta C, Cheng Z, de la Zerda A, Gheysens O, Gambhir SS (2008) Noninvasive molecular imaging of small living subjects using Raman spectroscopy. Proc Natl Acad Sci U S A 105:5844–5849
141. Zavaleta C, de la Zerda A, Liu Z, Keren S, Cheng Z, Schipper M, Chen X, Dai H, Gambhir SS (2008) Noninvasive Raman spectroscopy in living mice for evaluation of tumor targeting with carbon nanotubes. Nano Lett 8:2800–2805
142. Liang F, Chen B (2010) A review on biomedical applications of single-walled carbon nanotubes. Curr Med Chem 17:10–24
143. Mehra NK, Jain AK, Lodhi N, Raj R, Dubey V, Mishra D, Nahar M, Jain NK (2008) Challenges in the use of carbon nanotubes for biomedical applications. Crit Rev Ther Drug Carrier Syst 25:169–206
144. Shvedova AA, Kisin ER, Mercer R, Murray AR, Johnson VJ, Potapovich AI, Tyurina YY, Gorelik O, Arepalli S, Schwegler-Berry D, Hubbs AF, Antonini J, Evans DE, Ku BK, Ramsey D, Maynard A, Kagan VE, Castranova V, Baron P (2005) Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am J Physiol Lung Cell Mol Physiol 289:L698–L708
145. Kolosnjaj J, Szwarc H, Moussa F (2007) Toxicity studies of carbon nanotubes. Adv Exp Med Biol 620:181–204
146. Firme CP, Bandaru PR (2010) Toxicity issues in the application of carbon nanotubes to biological systems. Nanomedicine 6:245–256
147. Di Sotto A, Chiaretti M, Carru GA, Bellucci S, Mazzanti G (2009) Multi-walled carbon nanotubes: lack of mutagenic activity in the bacterial reverse mutation assay. Toxicol Lett 184:192–197
148. Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY, Riviere JE (2005) Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicol Lett 155:377–384
149. Cui D, Tian F, Ozkan CS, Wang M, Gao H (2005) Effect of single wall carbon nanotubes on human HEK293 cells. Toxicol Lett 155:73–85
150. Manna SK, Sarkar S, Barr J, Wise K, Barrera EV, Jejelowo O, Rice-Ficht AC, Ramesh GT (2005) Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes. Nano Lett 5:1676–1684

151. Sayes CM, Liang F, Hudson JL, Mendez J, Guo W, Beach JM, Moore VC, Doyle CD, West JL, Billups WE, Ausman KD, Colvin VL (2006) Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. Toxicol Lett 161:135–142
152. Bottini M, Bruckner S, Nika K, Bottini N, Bellucci S, Magrini A, Bergamaschi A, Mustelin T (2006) Multi-walled carbon nanotubes induce T lymphocyte apoptosis. Toxicol Lett 160:121–126
153. Ravichandran P, Baluchamy S, Sadanandan B, Gopikrishnan R, Biradar S, Ramesh V, Hall JC, Ramesh GT (2010) Multiwalled carbon nanotubes activate NF-kappaB and AP-1 signaling pathways to induce apoptosis in rat lung epithelial cells. Apoptosis: an international journal on programmed cell death 15:1507–1516
154. Patlolla A, Knighten B, Tchounwou P (2010) Multi-walled carbon nanotubes induce cytotoxicity, genotoxicity and apoptosis in normal human dermal fibroblast cells. Ethn Dis 20: S1-65–72
155. Bai Y, Zhang Y, Zhang J, Mu Q, Zhang W, Butch ER, Snyder SE, Yan B (2010) Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. Nat Nanotechnol 5:683–689
156. Sharma CS, Sarkar S, Periyakaruppan A, Barr J, Wise K, Thomas R, Wilson BL, Ramesh GT (2007) Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. J Nanosci Nanotechnol 7:2466–2472
157. Lam CW, James JT, McCluskey R, Arepalli S, Hunter RL (2006) A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. Crit Rev Toxicol 36:189–217
158. Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A (2006) Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol Sci 92:5–22
159. Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GA, Webb TR (2004) Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol Sci 77:117–125
160. Shvedova AA, Kisin ER, Murray AR, Gorelik O, Arepalli S, Castranova V, Young SH, Gao F, Tyurina YY, Oury TD, Kagan VE (2007) Vitamin E deficiency enhances pulmonary inflammatory response and oxidative stress induced by single-walled carbon nanotubes in C57BL/6 mice. Toxicol Appl Pharmacol 221:339–348
161. Muller J, Huaux F, Moreau N, Misson P, Heilier JF, Delos M, Arras M, Fonseca A, Nagy JB, Lison D (2005) Respiratory toxicity of multi-wall carbon nanotubes. Toxicol Appl Pharmacol 207:221–231
162. Kagan VE, Konduru NV, Feng W, Allen BL, Conroy J, Volkov Y, Vlasova II, Belikova NA, Yanamala N, Kapralov A, Tyurina YY, Shi J, Kisin ER, Murray AR, Franks J, Stolz D, Gou P, Klein-Seetharaman J, Fadeel B, Star A, Shvedova AA (2010) Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. Nat Nanotechnol 5:354–359
163. Folkmann JK, Risom L, Jacobsen NR, Wallin H, Loft S, Moller P (2009) Oxidatively damaged DNA in rats exposed by oral gavage to C60 fullerenes and single-walled carbon nanotubes. Environ Health Perspect 117:703–708
164. Kolosnjaj-Tabi J, Hartman KB, Boudjemaa S, Ananta JS, Morgant G, Szwarc H, Wilson LJ, Moussa F (2010) In vivo behavior of large doses of ultrashort and full-length single-walled carbon nanotubes after oral and intraperitoneal administration to swiss mice. ACS Nano 4:1481–1492
165. Ruggiero A, Villa CH, Bander E, Rey DA, Bergkvist M, Batt CA, Manova-Todorova K, Deen WM, Scheinberg DA, McDevitt MR (2010) Paradoxical glomerular filtration of carbon nanotubes. Proc Natl Acad Sci U S A 107:12369–12374
166. Cherukuri P, Gannon CJ, Leeuw TK, Schmidt HK, Smalley RE, Curley SA, Weisman RB (2006) Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence. Proc Natl Acad Sci U S A 103:18882–18886

167. Zhang D, Deng X, Ji Z, Shen X, Dong L, Wu M, Gu T, Liu Y (2010) Long-term hepatotoxicity of polyethylene-glycol functionalized multi-walled carbon nanotubes in mice. Nanotechnology 21:175101
168. Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, Stone V, Brown S, Macnee W, Donaldson K (2008) Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol 3:423–428
169. Yang ST, Wang X, Jia G, Gu Y, Wang T, Nie H, Ge C, Wang H, Liu Y (2008) Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice. Toxicol Lett 181:182–189
170. Semberova J, De Paoli Lacerda SH, Simakova O, Holada K, Gelderman MP, Simak J (2009) Carbon nanotubes activate blood platelets by inducing extracellular Ca2+ influx sensitive to calcium entry inhibitors. Nano Lett 9:3312–3317
171. Bihari P, Holzer M, Praetner M, Fent J, Lerchenberger M, Reichel CA, Rehberg M, Lakatos S, Krombach F (2010) Single-walled carbon nanotubes activate platelets and accelerate thrombus formation in the microcirculation. Toxicology 269:148–154
172. Thompson LC, Frasier CR, Sloan RC, Mann EE, Harrison BS, Brown JM, Brown DA, Wingard CJ (2014) Pulmonary instillation of multi-walled carbon nanotubes promotes coronary vasoconstriction and exacerbates injury in isolated hearts. Nanotoxicology 8:38–49. doi :10.3109/17435390.2012.744858
173. Stapleton PA, Minarchick VC, Cumpston AM, McKinney W, Chen BT, Sager TM, Frazer DG, Mercer RR, Scabilloni J, Andrew ME, Castranova V, Nurkiewicz TR (2012) Impairment of coronary arteriolar endothelium-dependent dilation after multi-walled carbon nanotube inhalation: a time-course study. Int J Mol Sci 13:13781–13803. doi:10.3390/ijms131113781, ijms131113781 [pii]
174. Garibaldi S, Brunelli C, Bavastrello V, Ghigliotti G, Nicolini C (2006) Carbon nanotube biocompatibility with cardiac muscle cells. Nanotechnology 17:391–397
175. Lin Z, Liu L, Xi Z, Huang J, Lin B (2012) Single-walled carbon nanotubes promote rat vascular adventitial fibroblasts to transform into myofibroblasts by SM22-alpha expression. Int J Nanomedicine 7:4199–4206. doi:10.2147/IJN.S34663, ijn-7-4199 [pii]
176. Martinelli V, Cellot G, Toma FM, Long CS, Caldwell JH, Zentilin L, Giacca M, Turco A, Prato M, Ballerini L, Mestroni L (2012) Carbon nanotubes promote growth and spontaneous electrical activity in cultured cardiac myocytes. Nano Lett 12:1831–1838. doi:10.1021/nl204064s
177. Zhiqing L, Zhuge X, Fuhuan C, Danfeng Y, Huashan Z, Bencheng L, Wei Z, Huanliang L, Xin S (2010) ICAM-1 and VCAM-1 expression in rat aortic endothelial cells after single-walled carbon nanotube exposure. J Nanosci Nanotechnol 10:8562–8574
178. Cheng WW, Lin ZQ, Ceng Q, Wei BF, Fan XJ, Zhang HS, Zhang W, Yang HL, Liu HL, Yan J, Tian L, Lin BC, Ding SM, Xi ZG (2012) Single-wall carbon nanotubes induce oxidative stress in rat aortic endothelial cells. Toxicol Mech Methods 22:268–276. doi:10.3109/15376516.2011.647112
179. Cheng WW, Lin ZQ, Wei BF, Zeng Q, Han B, Wei CX, Fan XJ, Hu CL, Liu LH, Huang JH, Yang X, Xi ZG (2011) Single-walled carbon nanotube induction of rat aortic endothelial cell apoptosis: reactive oxygen species are involved in the mitochondrial pathway. Int J Biochem Cell Biol 43:564–572. doi:10.1016/j.biocel.2010.12.013, S1357-2725(10)00425-5 [pii]
180. Xu YY, Yang J, Shen T, Zhou F, Xia Y, Fu JY, Meng J, Zhang J, Zheng YF, Xu LH, Zhu XQ (2012) Intravenous administration of multi-walled carbon nanotubes affects the formation of atherosclerosis in Sprague-Dawley rats. J Occup Health 54:361–369, doi:DN/JST.JSTAGE/joh/12-0019-OA [pii]
181. Cao Y, Jacobsen NR, Danielsen PH, Lenz AG, Stoeger T, Loft S, Wallin H, Roursgaard M, Mikkelsen L, Moller P (2014) Vascular effects of multiwalled carbon nanotubes in dyslipidemic ApoE-/- mice and cultured endothelial cells. Toxicol Sci 138:104–116. doi:10.1093/toxsci/kft328, kft328 [pii]
182. Ge C, Meng L, Xu L, Bai R, Du J, Zhang L, Li Y, Chang Y, Zhao Y, Chen C (2012) Acute pulmonary and moderate cardiovascular responses of spontaneously hypertensive rats after exposure to single-wall carbon nanotubes. Nanotoxicology 6:526–542. doi:10.3109/17435390.2011.587905

183. Urankar RN, Lust RM, Mann E, Katwa P, Wang X, Podila R, Hilderbrand SC, Harrison BS, Chen P, Ke PC, Rao AM, Brown JM, Wingard CJ (2012) Expansion of cardiac ischemia/reperfusion injury after instillation of three forms of multi-walled carbon nanotubes. Part Fibre Toxicol 9:38. doi:10.1186/1743-8977-9-38, 1743-8977-9-38 [pii]
184. Wang B, Feng W, Zhu M, Wang Y, Wang M, Gu Y, Ouyang H, Wang H, Li M, Zhao Y, Chai Z, Wang H (2009) Neurotoxicity of low-dose repeatedly intranasal instillation of nano- and submicron-sized ferric oxide particles in mice. J Nanopart Res 11:41–53
185. Wang J, Chen C, Liu Y, Jiao F, Li W, Lao F, Li Y, Li B, Ge C, Zhou G, Gao Y, Zhao Y, Chai Z (2008) Potential neurological lesion after nasal instillation of TiO2 nanoparticles in the anatase and rutile crystal phases. Toxicol Lett 183:72–80
186. Zhang Y, Ali SF, Dervishi E, Xu Y, Li Z, Casciano D, Biris AS (2010) Cytotoxicity effects of graphene and single-wall carbon nanotubes in neural phaeochromocytoma-derived PC12 cells. ACS Nano 4:3181–3186. doi:10.1021/nn1007176
187. Wang J, Sun P, Bao Y, Liu J, An L (2011) Cytotoxicity of single-walled carbon nanotubes on PC12 cells. Toxicol In Vitro 25:242–250. doi:10.1016/j.tiv.2010.11.010, S0887-2333(10)00302-4 [pii]
188. Zhang Y, Xu Y, Li Z, Chen T, Lantz SM, Howard PC, Paule MG, Slikker W Jr, Watanabe F, Mustafa T, Biris AS, Ali SF (2011) Mechanistic toxicity evaluation of uncoated and PEGylated single-walled carbon nanotubes in neuronal PC12 cells. ACS Nano 5:7020–7033. doi:10.1021/nn2016259
189. Wang J, Sun P, Bao Y, Dou B, Song D, Li Y (2012) Vitamin E renders protection to PC12 cells against oxidative damage and apoptosis induced by single-walled carbon nanotubes. Toxicol In Vitro 26:32–41. doi:10.1016/j.tiv.2011.10.004, S0887-2333(11)00270-0 [pii]
190. Xu H, Bai J, Meng J, Hao W, Cao JM (2009) Multi-walled carbon nanotubes suppress potassium channel activities in PC12 cells. Nanotechnology 20:285102. doi:10.1088/0957-4484/20/28/285102, S0957-4484(09)06963-3 [pii]
191. Meng L, Chen R, Jiang A, Wang L, Wang P, Li CZ, Bai R, Zhao Y, Autrup H, Chen C (2013) Short multiwall carbon nanotubes promote neuronal differentiation of PC12 cells via up-regulation of the neurotrophin signaling pathway. Small 9:1786–1798. doi:10.1002/smll.201201388
192. Gavello D, Vandael DH, Cesa R, Premoselli F, Marcantoni A, Cesano F, Scarano D, Fubini B, Carbone E, Fenoglio I, Carabelli V (2012) Altered excitability of cultured chromaffin cells following exposure to multi-walled carbon nanotubes. Nanotoxicology 6:47–60. doi:10.3109/17435390.2011.553294
193. Varro P, Szigyarto IC, Gergely A, Kalman E, Vilagi I (2013) Carbon nanotubes exert basic excitatory enhancement in rat brain slices. Acta Biol Hung 64:137–151. doi:10.1556/ABiol.64.2013.2.1, 42602421T87W4605 [pii]
194. Chen T, Yang J, Ren G, Yang Z, Zhang T (2013) Multi-walled carbon nanotube increases the excitability of hippocampal CA1 neurons through inhibition of potassium channels in rat's brain slices. Toxicol Lett 217:121–128. doi:10.1016/j.toxlet.2012.12.013, S0378-4274(12)01442-7 [pii]
195. Gladwin KM, Whitby RL, Mikhalovsky SV, Tomlins P, Adu J (2013) In vitro biocompatibility of multiwalled carbon nanotubes with sensory neurons. Adv Healthc Mater 2:728–735. doi:10.1002/adhm.201200233
196. Wu D, Pak ES, Wingard CJ, Murashov AK (2012) Multi-walled carbon nanotubes inhibit regenerative axon growth of dorsal root ganglia neurons of mice. Neurosci Lett 507:72–77. doi:10.1016/j.neulet.2011.11.056, S0304-3940(11)01578-3 [pii]
197. Han YG, Xu J, Li ZG, Ren GG, Yang Z (2012) In vitro toxicity of multi-walled carbon nanotubes in C6 rat glioma cells. Neurotoxicology 33:1128–1134. doi:10.1016/j.neuro.2012.06.004, S0161-813X(12)00136-2 [pii]
198. Matsumoto K, Sato C, Naka Y, Whitby R, Shimizu N (2010) Stimulation of neuronal neurite outgrowth using functionalized carbon nanotubes. Nanotechnology 21:115101. doi:10.1088/0957-4484/21/11/115101, S0957-4484(10)37990-6 [pii]

199. Ivani S, Karimi I, Tabatabaei SR (2012) Biosafety of multiwalled carbon nanotube in mice: a behavioral toxicological approach. J Toxicol Sci 37:1191–1205, doi:DN/JST.JSTAGE/jts/37.1191 [pii]
200. Bhirde AA, Patel S, Sousa AA, Patel V, Molinolo AA, Ji Y, Leapman RD, Gutkind JS, Rusling JF (2010) Distribution and clearance of PEG-single-walled carbon nanotube cancer drug delivery vehicles in mice. Nanomedicine (Lond) 5:1535–1546. doi:10.2217/nnm.10.90
201. Wang X, Podila R, Shannahan JH, Rao AM, Brown JM (2013) Intravenously delivered graphene nanosheets and multiwalled carbon nanotubes induce site-specific Th2 inflammatory responses via the IL-33/ST2 axis. Int J Nanomedicine 8:1733–1748. doi:10.2147/IJN.S44211, ijn-8-1733 [pii]
202. Meng J, Yang M, Jia F, Xu Z, Kong H, Xu H (2011) Immune responses of BALB/c mice to subcutaneously injected multi-walled carbon nanotubes. Nanotoxicology 5:583–591. doi:10.3109/17435390.2010.523483
203. Sato Y, Yokoyama A, Nodasaka Y, Kohgo T, Motomiya K, Matsumoto H, Nakazawa E, Numata T, Zhang M, Yudasaka M, Hara H, Araki R, Tsukamoto O, Saito H, Kamino T, Watari F, Tohji K (2013) Long-term biopersistence of tangled oxidized carbon nanotubes inside and outside macrophages in rat subcutaneous tissue. Sci Rep 3:2516. doi:10.1038/srep02516, srep02516 [pii]
204. Pinto NV, de Andrade NF, Martinez DS, Alves OL, Souza Filho AG, Mota MR, Nascimento KS, Cavada BS, Assreuy AM (2013) Inflammatory and hyperalgesic effects of oxidized multi-walled carbon nanotubes in rats. J Nanosci Nanotechnol 13:5276–5282
205. Trpkovic A, Todorovic-Markovic B, Trajkovic V (2012) Toxicity of pristine versus functionalized fullerenes: mechanisms of cell damage and the role of oxidative stress. Arch Toxicol 86:1809–1827. doi:10.1007/s00204-012-0859-6
206. Dal Forno GO, Kist LW, de Azevedo MB, Fritsch RS, Pereira TC, Britto RS, Guterres SS, Kulkamp-Guerreiro IC, Bonan CD, Monserrat JM, Bogo MR (2013) Intraperitoneal exposure to nano/microparticles of fullerene (C(6)(0)) increases acetylcholinesterase activity and lipid peroxidation in adult zebrafish (Danio rerio) brain. Biomed Res Int 2013:1–11
207. Ferreira JL, Lonne MN, Franca TA, Maximilla NR, Lugokenski TH, Costa PG, Fillmann G, Antunes Soares FA, de la Torre FR, Monserrat JM (2014) Co-exposure of the organic nanomaterial fullerene C(6)(0) with benzo[a]pyrene in Danio rerio (zebrafish) hepatocytes: evidence of toxicological interactions. Aquat Toxicol 147:76–83. doi:10.1016/j.aquatox.2013.12.007
208. Thompson LC, Urankar RN, Holland NA, Vidanapathirana AK, Pitzer JE, Han L, Sumner SJ, Lewin AH, Fennell TR, Lust RM, Brown JM, Wingard CJ (2014) C(6)(0) exposure augments cardiac ischemia/reperfusion injury and coronary artery contraction in Sprague Dawley rats. Toxicol Sci 138:365–378. doi:10.1093/toxsci/kfu008
209. Goncalves DM, Girard D (2013) Evidence that polyhydroxylated C60 fullerenes (fullerenols) amplify the effect of lipopolysaccharides to induce rapid leukocyte infiltration in vivo. Chem Res Toxicol 26:1884–1892. doi:10.1021/tx4002622
210. Kalaria DR, Sharma G, Beniwal V, Ravi Kumar MN (2009) Design of biodegradable nanoparticles for oral delivery of doxorubicin: in vivo pharmacokinetics and toxicity studies in rats. Pharm Res 26:492–501
211. Rytting E, Nguyen J, Wang X, Kissel T (2008) Biodegradable polymeric nanocarriers for pulmonary drug delivery. Expert Opin Drug Deliv 5:629–639
212. Wilson B, Samanta MK, Santhi K, Kumar KP, Ramasamy M, Suresh B (2010) Chitosan nanoparticles as a new delivery system for the anti-Alzheimer drug tacrine. Nanomedicine 6:144–152
213. Anand P, Nair HB, Sung B, Kunnumakkara AB, Yadav VR, Tekmal RR, Aggarwal BB (2010) Design of curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. Biochem Pharmacol 79:330–338

214. Nanjwade BK, Singh J, Parikh KA, Manvi FV (2010) Preparation and evaluation of carboplatin biodegradable polymeric nanoparticles. Int J Pharm 385:176–180
215. Jain RA (2000) The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials 21:2475–2490
216. Cheng FY, Wang SP, Su CH, Tsai TL, Wu PC, Shieh DB, Chen JH, Hsieh PC, Yeh CS (2008) Stabilizer-free poly(lactide-co-glycolide) nanoparticles for multimodal biomedical probes. Biomaterials 29:2104–2112
217. Nehilla BJ, Allen PG, Desai TA (2008) Surfactant-free, drug-quantum-dot coloaded poly(lactide-co-glycolide) nanoparticles: towards multifunctional nanoparticles. ACS Nano 2:538–544
218. Park H, Yang J, Lee J, Haam S, Choi IH, Yoo KH (2009) Multifunctional nanoparticles for combined doxorubicin and photothermal treatments. ACS Nano 3:2919–2926
219. Betancourt T, Brown B, Brannon-Peppas L (2007) Doxorubicin-loaded PLGA nanoparticles by nanoprecipitation: preparation, characterization and in vitro evaluation. Nanomedicine (Lond) 2:219–232
220. Dong Y, Feng SS (2007) Poly(D, L-lactide-co-glycolide) (PLGA) nanoparticles prepared by high pressure homogenization for paclitaxel chemotherapy. Int J Pharm 342:208–214
221. Lee S, Ryu JH, Park K, Lee A, Lee SY, Youn IC, Ahn CH, Yoon SM, Myung SJ, Moon DH, Chen X, Choi K, Kwon IC, Kim K (2009) Polymeric nanoparticle-based activatable near-infrared nanosensor for protease determination in vivo. Nano Lett 9:4412–4416
222. Myc A, Kukowska-Latallo J, Cao P, Swanson B, Battista J, Dunham T, Baker JR (2010) Targeting the efficacy of a dendrimer-based nanotherapeutic in heterogeneous xenograft tumors in vivo. Anticancer Drugs 21:186–192
223. Liu M, Fu J, Li J, Wang L, Tan Q, Ren X, Peng Z, Zeng H (2010) Preparation of tri-block copolymer micelles loading novel organoselenium anticancer drug BBSKE and study of tissue distribution of copolymer micelles by imaging in vivo method. Int J Pharm 391:292–304
224. Xu Y, Wen Z, Xu Z (2009) Chitosan nanoparticles inhibit the growth of human hepatocellular carcinoma xenografts through an antiangiogenic mechanism. Anticancer Res 29:5103–5109
225. Wang CX, Huang LS, Hou LB, Jiang L, Yan ZT, Wang YL, Chen ZL (2009) Antitumor effects of polysorbate-80 coated gemcitabine polybutylcyanoacrylate nanoparticles in vitro and its pharmacodynamics in vivo on C6 glioma cells of a brain tumor model. Brain Res 1261:91–99
226. Cenni E, Granchi D, Avnet S, Fotia C, Salerno M, Micieli D, Sarpietro MG, Pignatello R, Castelli F, Baldini N (2008) Biocompatibility of poly(D, L-lactide-co-glycolide) nanoparticles conjugated with alendronate. Biomaterials 29:1400–1411
227. Kabanov AV (2006) Polymer genomics: an insight into pharmacology and toxicology of nanomedicines. Adv Drug Deliv Rev 58:1597–1621
228. Kawaguchi T, Honda T, Nishihara M, Yamamoto T, Yokoyama M (2009) Histological study on side effects and tumor targeting of a block copolymer micelle on rats. J Control Release 136:240–246
229. Chen Z, Chen H, Meng H, Xing G, Gao X, Sun B, Shi X, Yuan H, Zhang C, Liu R, Zhao F, Zhao Y, Fang X (2008) Bio-distribution and metabolic paths of silica coated CdSeS quantum dots. Toxicol Appl Pharmacol 230:364–371
230. Gao X, Yang L, Petros JA, Marshall FF, Simons JW, Nie S (2005) In vivo molecular and cellular imaging with quantum dots. Curr Opin Biotechnol 16:63–72
231. Iga AM, Robertson JH, Winslet MC, Seifalian AM (2007) Clinical potential of quantum dots. J Biomed Biotechnol 2007:76087
232. Cai W, Hsu AR, Li ZB, Chen X (2007) Are quantum dots ready for in vivo imaging in human subjects? Nanoscale Res Lett 2:265–281
233. Larson DR, Zipfel WR, Williams RM, Clark SW, Bruchez MP, Wise FW, Webb WW (2003) Water-soluble quantum dots for multiphoton fluorescence imaging in vivo. Science 300:1434–1436

234. Voura EB, Jaiswal JK, Mattoussi H, Simon SM (2004) Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. Nat Med 10:993–998
235. Jaiswal JK, Mattoussi H, Mauro JM, Simon SM (2003) Long-term multiple color imaging of live cells using quantum dot bioconjugates. Nat Biotechnol 21:47–51
236. Schroeder JE, Shweky I, Shmeeda H, Banin U, Gabizon A (2007) Folate-mediated tumor cell uptake of quantum dots entrapped in lipid nanoparticles. J Control Release 124:28–34
237. Akerman ME, Chan WC, Laakkonen P, Bhatia SN, Ruoslahti E (2002) Nanocrystal targeting in vivo. Proc Natl Acad Sci U S A 99:12617–12621
238. So MK, Xu C, Loening AM, Gambhir SS, Rao J (2006) Self-illuminating quantum dot conjugates for in vivo imaging. Nat Biotechnol 24:339–343
239. Al-Jamal WT, Al-Jamal KT, Tian B, Cakebread A, Halket JM, Kostarelos K (2009) Tumor targeting of functionalized quantum dot-liposome hybrids by intravenous administration. Mol Pharm 6:520–530
240. Pan J, Feng SS (2009) Targeting and imaging cancer cells by folate-decorated, quantum dots (QDs)- loaded nanoparticles of biodegradable polymers. Biomaterials 30:1176–1183
241. Lin P, Chen JW, Chang LW, Wu JP, Redding L, Chang H, Yeh TK, Yang CS, Tsai MH, Wang HJ, Kuo YC, Yang RS (2008) Computational and ultrastructural toxicology of a nanoparticle, Quantum Dot 705, in mice. Environ Sci Technol 42:6264–6270
242. Mahto SK, Park C, Yoon TH, Rhee SW (2010) Assessment of cytocompatibility of surface-modified CdSe/ZnSe quantum dots for BALB/3T3 fibroblast cells. Toxicol In Vitro 24:1070–1077
243. Hardman R (2006) A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environ Health Perspect 114:165–172
244. Wang L, Zheng H, Long Y, Gao M, Hao J, Du J, Mao X, Zhou D (2010) Rapid determination of the toxicity of quantum dots with luminous bacteria. J Hazard Mater 177:1134–1137
245. Khatchadourian A, Maysinger D (2009) Lipid droplets: their role in nanoparticle-induced oxidative stress. Mol Pharm 6:1125–1137
246. Hauck TS, Anderson RE, Fischer HC, Newbigging S, Chan WC (2010) In vivo quantum-dot toxicity assessment. Small 6:138–144
247. Pelley JL, Daar AS, Saner MA (2009) State of academic knowledge on toxicity and biological fate of quantum dots. Toxicol Sci 112:276–296
248. Lovric J, Cho SJ, Winnik FM, Maysinger D (2005) Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. Chem Biol 12:1227–1234
249. Cho SJ, Maysinger D, Jain M, Roder B, Hackbarth S, Winnik FM (2007) Long-term exposure to CdTe quantum dots causes functional impairments in live cells. Langmuir 23:1974–1980
250. Fitzpatrick JA, Andreko SK, Ernst LA, Waggoner AS, Ballou B, Bruchez MP (2009) Long-term persistence and spectral blue shifting of quantum dots in vivo. Nano Lett 9:2736–2741
251. Geys J, Nemmar A, Verbeken E, Smolders E, Ratoi M, Hoylaerts MF, Nemery B, Hoet PH (2008) Acute toxicity and prothrombotic effects of quantum dots: impact of surface charge. Environ Health Perspect 116:1607–1613
252. Pons T, Pic E, Lequeux N, Cassette E, Bezdetnaya L, Guillemin F, Marchal F, Dubertret B (2010) Cadmium-free CuInS2/ZnS quantum dots for sentinel lymph node imaging with reduced toxicity. ACS Nano 4:2531–2538
253. Tseng MT, Lu X, Duan X, Hardas SS, Sultana R, Wu P, Unrine JM, Graham U, Butterfield DA, Grulke EA, Yokel RA (2012) Alteration of hepatic structure and oxidative stress induced by intravenous nanoceria. Toxicol Appl Pharmacol 260:173–182. doi:10.1016/j.taap.2012.02.008
254. Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F (2009) Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations–many questions, some answers. Mutat Res 681:241–258

255. Manke A, Wang L, Rojanasakul Y (2013) Mechanisms of nanoparticle-induced oxidative stress and toxicity. Biomed Res Int 2013:942916. doi:10.1155/2013/942916
256. Roesslein M, Hirsch C, Kaiser JP, Krug HF, Wick P (2013) Comparability of in vitro tests for bioactive nanoparticles: a common assay to detect reactive oxygen species as an example. Int J Mol Sci 14:24320–24337. doi:10.3390/ijms141224320
257. Karlsson HL, Cronholm P, Gustafsson J, Moller L (2008) Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol 21:1726–1732
258. He L, Yang L, Zhang ZR, Gong T, Deng L, Gu Z, Sun X (2009) In vitro evaluation of the genotoxicity of a family of novel MeO-PEG-poly(D, L-lactic-co-glycolic acid)-PEG-OMe triblock copolymer and PLGA nanoparticles. Nanotechnology 20:455102
259. Barnes CA, Elsaesser A, Arkusz J, Smok A, Palus J, Lesniak A, Salvati A, Hanrahan JP, Jong WH, Dziubaltowska E, Stepnik M, Rydzynski K, McKerr G, Lynch I, Dawson KA, Howard CV (2008) Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. Nano Lett 8:3069–3074
260. Jin Y, Kannan S, Wu M, Zhao JX (2007) Toxicity of luminescent silica nanoparticles to living cells. Chem Res Toxicol 20:1126–1133
261. Shinohara N, Matsumoto K, Endoh S, Maru J, Nakanishi J (2009) In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. Toxicol Lett 191:289–296
262. Mori T, Takada H, Ito S, Matsubayashi K, Miwa N, Sawaguchi T (2006) Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. Toxicology 225:48–54
263. Wirnitzer U, Herbold B, Voetz M, Ragot J (2009) Studies on the in vitro genotoxicity of baytubes, agglomerates of engineered multi-walled carbon-nanotubes (MWCNT). Toxicol Lett 186:160–165
264. Zhu L, Chang DW, Dai L, Hong Y (2007) DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells. Nano Lett 7:3592–3597
265. Dhawan A, Taurozzi JS, Pandey AK, Shan W, Miller SM, Hashsham SA, Tarabara VV (2006) Stable colloidal dispersions of C60 fullerenes in water: evidence for genotoxicity. Environ Sci Technol 40:7394–7401
266. Chen M, von Mikecz A (2005) Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 nanoparticles. Exp Cell Res 305:51–62
267. AshaRani PV, Low Kah Mun G, Hande MP, Valiyaveettil S (2009) Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano 3:279–290
268. An H, Liu Q, Ji Q, Jin B (2010) DNA binding and aggregation by carbon nanoparticles. Biochem Biophys Res Commun 393:571–576
269. Park EJ, Yi J, Chung KH, Ryu DY, Choi J, Park K (2008) Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicol Lett 180:222–229
270. Barillet S, Jugan ML, Laye M, Leconte Y, Herlin-Boime N, Reynaud C, Carriere M (2010) In vitro evaluation of SiC nanoparticles impact on A549 pulmonary cells: cyto-, genotoxicity and oxidative stress. Toxicol Lett 198:324–330
271. Kisin ER, Murray AR, Keane MJ, Shi XC, Schwegler-Berry D, Gorelik O, Arepalli S, Castranova V, Wallace WE, Kagan VE, Shvedova AA (2007) Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells. J Toxicol Environ Health A 70:2071–2079
272. Muller J, Decordier I, Hoet PH, Lombaert N, Thomassen L, Huaux F, Lison D, Kirsch-Volders M (2008) Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. Carcinogenesis 29:427–433
273. Huang S, Chueh PJ, Lin YW, Shih TS, Chuang SM (2009) Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO2 long-term exposure. Toxicol Appl Pharmacol 241:182–194

274. Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH (2009) Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res 69:8784–8789
275. Hackenberg S, Friehs G, Kessler M, Froelich K, Ginzkey C, Koehler C, Scherzed A, Burghartz M, Kleinsasser N (2011) Nanosized titanium dioxide particles do not induce DNA damage in human peripheral blood lymphocytes. Environ Mol Mutagen 52(4):264–268
276. Mrdanovic J, Solajic S, Bogdanovic V, Stankov K, Bogdanovic G, Djordjevic A (2009) Effects of fullerenol C60(OH)24 on the frequency of micronuclei and chromosome aberrations in CHO-K1 cells. Mutat Res 680:25–30
277. Pardridge WM (2005) The blood-brain barrier: bottleneck in brain drug development. NeuroRx 2:3–14
278. Gao X, Chen J, Chen J, Wu B, Chen H, Jiang X (2008) Quantum dots bearing lectin-functionalized nanoparticles as a platform for in vivo brain imaging. Bioconjug Chem 19:2189–2195
279. Fernandes C, Soni U, Patravale V (2010) Nano-interventions for neurodegenerative disorders. Pharmacol Res 62:166–178
280. Kabanov AV, Gendelman HE (2007) Nanomedicine in the diagnosis and therapy of neurodegenerative disorders. Prog Polym Sci 32:1054–1082
281. Yang H (2010) Nanoparticle-mediated brain-specific drug delivery, imaging, and diagnosis. Pharm Res 27:1759–1771
282. Gelperina S, Maksimenko O, Khalansky A, Vanchugova L, Shipulo E, Abbasova K, Berdiev R, Wohlfart S, Chepurnova N, Kreuter J (2010) Drug delivery to the brain using surfactant-coated poly(lactide-co-glycolide) nanoparticles: influence of the formulation parameters. Eur J Pharm Biopharm 74:157–163
283. Wong HL, Chattopadhyay N, Wu XY, Bendayan R (2010) Nanotechnology applications for improved delivery of antiretroviral drugs to the brain. Adv Drug Deliv Rev 62:503–517
284. Tosi G, Costantino L, Rivasi F, Ruozi B, Leo E, Vergoni AV, Tacchi R, Bertolini A, Vandelli MA, Forni F (2007) Targeting the central nervous system: in vivo experiments with peptide-derivatized nanoparticles loaded with Loperamide and Rhodamine-123. J Control Release 122:1–9
285. Reimold I, Domke D, Bender J, Seyfried CA, Radunz HE, Fricker G (2008) Delivery of nanoparticles to the brain detected by fluorescence microscopy. Eur J Pharm Biopharm 70:627–632
286. Oberdorster E (2004) Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. Environ Health Perspect 112:1058–1062
287. Mistry A, Stolnik S, Illum L (2009) Nanoparticles for direct nose-to-brain delivery of drugs. Int J Pharm 379:146–157
288. Kreuter J (2004) Influence of the surface properties on nanoparticle-mediated transport of drugs to the brain. J Nanosci Nanotechnol 4:484–488
289. Steiniger SC, Kreuter J, Khalansky AS, Skidan IN, Bobruskin AI, Smirnova ZS, Severin SE, Uhl R, Kock M, Geiger KD, Gelperina SE (2004) Chemotherapy of glioblastoma in rats using doxorubicin-loaded nanoparticles. Int J Cancer 109:759–767
290. Alyautdin RN, Petrov VE, Langer K, Berthold A, Kharkevich DA, Kreuter J (1997) Delivery of loperamide across the blood-brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. Pharm Res 14:325–328
291. Kreuter J, Gelperina S (2008) Use of nanoparticles for cerebral cancer. Tumori 94:271–277
292. Kurakhmaeva KB, Djindjikhashvili IA, Petrov VE, Balabanyan VU, Voronina TA, Trofimov SS, Kreuter J, Gelperina S, Begley D, Alyautdin RN (2009) Brain targeting of nerve growth factor using poly(butyl cyanoacrylate) nanoparticles. J Drug Target 17:564–574
293. Petri B, Bootz A, Khalansky A, Hekmatara T, Muller R, Uhl R, Kreuter J, Gelperina S (2007) Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated poly(butyl cyanoacrylate) nanoparticles: revisiting the role of surfactants. J Control Release 117:51–58
294. Sun W, Xie C, Wang H, Hu Y (2004) Specific role of polysorbate 80 coating on the targeting of nanoparticles to the brain. Biomaterials 25:3065–3071

295. Wilson B, Samanta MK, Santhi K, Kumar KP, Paramakrishnan N, Suresh B (2008) Poly(n-butylcyanoacrylate) nanoparticles coated with polysorbate 80 for the targeted delivery of rivastigmine into the brain to treat Alzheimer's disease. Brain Res 1200:159–168
296. Mulik RS, Monkkonen J, Juvonen RO, Mahadik KR, Paradkar AR (2010) ApoE3 mediated poly(butyl) cyanoacrylate nanoparticles containing curcumin: study of enhanced activity of curcumin against beta amyloid induced cytotoxicity using in vitro cell culture model. Mol Pharm 7:815–825
297. Zensi A, Begley D, Pontikis C, Legros C, Mihoreanu L, Wagner S, Buchel C, von Briesen H, Kreuter J (2009) Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and are delivered to neurones. J Control Release 137:78–86
298. Tosi G, Vergoni AV, Ruozi B, Bondioli L, Badiali L, Rivasi F, Costantino L, Forni F, Vandelli MA (2010) Sialic acid and glycopeptides conjugated PLGA nanoparticles for central nervous system targeting: in vivo pharmacological evidence and biodistribution. J Control Release 145:49–57
299. Pandey R, Khuller GK (2006) Oral nanoparticle-based antituberculosis drug delivery to the brain in an experimental model. J Antimicrob Chemother 57:1146–1152
300. Yang Z, Zhang Y, Yang Y, Sun L, Han D, Li H, Wang C (2010) Pharmacological and toxicological target organelles and safe use of single-walled carbon nanotubes as drug carriers in treating Alzheimer disease. Nanomedicine 6:427–441
301. Sharma HS, Hussain S, Schlager J, Ali SF, Sharma A (2010) Influence of nanoparticles on blood-brain barrier permeability and brain edema formation in rats. Acta Neurochir Suppl 106:359–364
302. Win-Shwe TT, Yamamoto S, Fujitani Y, Hirano S, Fujimaki H (2008) Spatial learning and memory function-related gene expression in the hippocampus of mouse exposed to nanoparticle-rich diesel exhaust. Neurotoxicology 29:940–947
303. Yang Z, Liu ZW, Allaker RP, Reip P, Oxford J, Ahmad Z, Ren G (2010) A review of nanoparticle functionality and toxicity on the central nervous system. J R Soc Interface 7(Suppl 4):S411–S422
304. Shimizu M, Tainaka H, Oba T, Mizuo K, Umezawa M, Takeda K (2009) Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse. Part Fibre Toxicol 6:20
305. Rahman MF, Wang J, Patterson TA, Saini UT, Robinson BL, Newport GD, Murdock RC, Schlager JJ, Hussain SM, Ali SF (2009) Expression of genes related to oxidative stress in the mouse brain after exposure to silver-25 nanoparticles. Toxicol Lett 187:15–21
306. Tang J, Xiong L, Wang S, Wang J, Liu L, Li J, Yuan F, Xi T (2009) Distribution, translocation and accumulation of silver nanoparticles in rats. J Nanosci Nanotechnol 9:4924–4932
307. Liu J, Hopfinger AJ (2008) Identification of possible sources of nanotoxicity from carbon nanotubes inserted into membrane bilayers using membrane interaction quantitative structure–activity relationship analysis. Chem Res Toxicol 21:459–466. doi:10.1021/tx700392b
308. Peter C, Hummer G (2005) Ion transport through membrane-spanning nanopores studied by molecular dynamics simulations and continuum electrostatics calculations. Biophys J 89:2222–2234. doi:10.1529/biophysj.105.065946, biophysj.105.065946 [pii]
309. Shimizu K, Uchiyama A, Yamashita M, Hirose A, Nishimura T, Oku N (2013) Biomembrane damage caused by exposure to multi-walled carbon nanotubes. J Toxicol Sci 38:7–12, doi:DN/JST.JSTAGE/jts/38.7 [pii]
310. Hirano S, Kanno S, Furuyama A (2008) Multi-walled carbon nanotubes injure the plasma membrane of macrophages. Toxicol Appl Pharmacol 232:244–251. doi:10.1016/j.taap.2008.06.016, S0041-008X(08)00271-8 [pii]
311. Hirano S, Fujitani Y, Furuyama A, Kanno S (2012) Macrophage receptor with collagenous structure (MARCO) is a dynamic adhesive molecule that enhances uptake of carbon nanotubes by CHO-K1 cells. Toxicol Appl Pharmacol 259:96–103. doi:10.1016/j.taap.2011.12.012, S0041-008X(11)00469-8 [pii]
312. Shvedova A, Castranova V, Kisin E, Schwegler-Berry D, Murray A, Gandelsman V, Maynard A, Baron P (2003) Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. J Toxicol Environ Health A 66:1909–1926

313. Pulskamp K, Diabate S, Krug HF (2007) Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. Toxicol Lett 168:58–74
314. Fenoglio I, Tomatis M, Lison D, Muller J, Fonseca A, Nagy JB, Fubini B (2006) Reactivity of carbon nanotubes: free radical generation or scavenging activity? Free Radic Biol Med 40:1227–1233. doi:10.1016/j.freeradbiomed.2005.11.010, S0891-5849(05)00708-2 [pii]
315. Zhu L, Chang DW, Dai L, Hong Y (2007) DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells. Nano Lett 7:3592–3597. doi:10.1021/nl071303v
316. Pacurari M, Yin XJ, Zhao J, Ding M, Leonard SS, Schwegler-Berry D, Ducatman BS, Sbarra D, Hoover MD, Castranova V, Vallyathan V (2008) Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells. Environ Health Perspect 116:1211–1217. doi:10.1289/ehp.10924
317. Choi SJ, Oh JM, Choy JH (2009) Toxicological effects of inorganic nanoparticles on human lung cancer A549 cells. J Inorg Biochem 103:463–471
318. Pulskamp K (2008) Influence of carbon nanoparticles on cell physiology - mechanistic studies on the toxic effects. Wissenschaftliche Berichte FZKA 7401: A-200
319. Jia G, Wang H, Yan L, Wang X, Pei R, Yan T, Zhao Y, Guo X (2005) Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene. Environ Sci Tech 39:1378–1383
320. Pacurari M, Yin XJ, Ding M, Leonard SS, Schwegler-Berry D, Ducatman BS, Chirila M, Endo M, Castranova V, Vallyathan V (2008) Oxidative and molecular interactions of multi-wall carbon nanotubes (MWCNT) in normal and malignant human mesothelial cells. Nanotoxicology 2:155–170
321. Sato Y, Shibata K, Kataoka H, Ogino S, Bunshi F, Yokoyama A, Tamura K, Akasaka T, Uo M, Motomiya K, Jeyadevan B, Hatakeyama R, Watari F, Tohji K (2005) Strict preparation and evaluation of water-soluble hat-stacked carbon nanofibers for biomedical application and their high biocompatibility: influence of nanofiber-surface functional groups on cytotoxicity. Mol Biosyst 1:142–145
322. Sarkar S, Sharma C, Yog R, Periakaruppan A, Jejelowo O, Thomas R, Barrera EV, Rice-Ficht AC, Wilson BL, Ramesh GT (2007) Analysis of stress responsive genes induced by single-walled carbon nanotubes in BJ foreskin cells. J Nanosci Nanotechnol 7:584–592
323. Witzmann FA, Monteiro-Riviere NA (2006) Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes. Nanomedicine 2:158–168. doi:10.1016/j.nano.2006.07.005, S1549-9634(06)00103-1 [pii]
324. Barna BP, Huizar I, Malur A, McPeek M, Marshall I, Jacob M, Dobbs L, Kavuru MS, Thomassen MJ (2013) Carbon nanotube-induced pulmonary granulomatous disease: Twist1 and alveolar macrophage M1 activation. Int J Mol Sci 14:23858–23871. doi:10.3390/ijms141223858, ijms141223858 [pii]
325. Beamer CA, Girtsman TA, Seaver BP, Finsaas KJ, Migliaccio CT, Perry VK, Rottman JB, Smith DE, Holian A (2013) IL-33 mediates multi-walled carbon nanotube (MWCNT)-induced airway hyper-reactivity via the mobilization of innate helper cells in the lung. Nanotoxicology 7:1070–1081. doi:10.3109/17435390.2012.702230
326. Ronzani C, Spiegelhalter C, Vonesch JL, Lebeau L, Pons F (2012) Lung deposition and toxicological responses evoked by multi-walled carbon nanotubes dispersed in a synthetic lung surfactant in the mouse. Arch Toxicol 86:137–149. doi:10.1007/s00204-011-0741-y
327. Sager TM, Wolfarth MW, Andrew M, Hubbs A, Friend S, Chen TH, Porter DW, Wu N, Yang F, Hamilton RF, Holian A (2014) Effect of multi-walled carbon nanotube surface modification on bioactivity in the C57BL/6 mouse model. Nanotoxicology 8:317–327. doi:10.3109/17435390.2013.779757
328. Park EJ, Roh J, Kim SN, Kim Y, Han SB, Hong JT (2013) CCR5 plays an important role in resolving an inflammatory response to single-walled carbon nanotubes. J Appl Toxicol 33:845–853. doi:10.1002/jat.2744

329. Park EJ, Cho WS, Jeong J, Yi J, Choi K, Park K (2009) Pro-inflammatory and potential allergic responses resulting from B cell activation in mice treated with multi-walled carbon nanotubes by intratracheal instillation. Toxicology 259:113–121
330. Chang CC, Tsai ML, Huang HC, Chen CY, Dai SX (2012) Epithelial-mesenchymal transition contributes to SWCNT-induced pulmonary fibrosis. Nanotoxicology 6:600–610. doi:10.3109/17435390.2011.594913
331. Huizar I, Malur A, Patel J, McPeek M, Dobbs L, Wingard C, Barna BP, Thomassen MJ (2013) The role of PPARgamma in carbon nanotube-elicited granulomatous lung inflammation. Respir Res 14:7. doi:10.1186/1465-9921-14-7, 1465-9921-14-7 [pii]
332. Girtsman TA, Beamer CA, Wu N, Buford M, Holian A (2014) IL-1R signalling is critical for regulation of multi-walled carbon nanotubes-induced acute lung inflammation in C57Bl/6 mice. Nanotoxicology 8:17–27. doi:10.3109/17435390.2012.744110
333. Di YP, Tkach AV, Yanamala N, Stanley S, Gao S, Shurin MR, Kisin ER, Kagan VE, Shvedova A (2013) Dual acute proinflammatory and antifibrotic pulmonary effects of short palate, lung, and nasal epithelium clone-1 after exposure to carbon nanotubes. Am J Respir Cell Mol Biol 49:759–767. doi:10.1165/rcmb.2012-0435OC
334. Snyder-Talkington BN, Dymacek J, Porter DW, Wolfarth MG, Mercer RR, Pacurari M, Denvir J, Castranova V, Qian Y, Guo NL (2013) System-based identification of toxicity pathways associated with multi-walled carbon nanotube-induced pathological responses. Toxicol Appl Pharmacol 272:476–489. doi:10.1016/j.taap.2013.06.026, S0041-008X(13)00299-8 [pii]
335. Snyder-Talkington BN, Pacurari M, Dong C, Leonard SS, Schwegler-Berry D, Castranova V, Qian Y, Guo NL (2013) Systematic analysis of multiwalled carbon nanotube-induced cellular signaling and gene expression in human small airway epithelial cells. Toxicol Sci 133:79–89. doi:10.1093/toxsci/kft019, kft019 [pii]
336. Tyurina YY, Kisin ER, Murray A, Tyurin VA, Kapralova VI, Sparvero LJ, Amoscato AA, Samhan-Arias AK, Swedin L, Lahesmaa R, Fadeel B, Shvedova AA, Kagan VE (2011) Global phospholipidomics analysis reveals selective pulmonary peroxidation profiles upon inhalation of single-walled carbon nanotubes. ACS Nano 5:7342–7353. doi:10.1021/nn202201j
337. Wang L, Mercer RR, Rojanasakul Y, Qiu A, Lu Y, Scabilloni JF, Wu N, Castranova V (2010) Direct fibrogenic effects of dispersed single-walled carbon nanotubes on human lung fibroblasts. J Toxicol Environ Health A 73:410–422. doi:10.1080/15287390903486550, 919251280 [pii]
338. Wang X, Shannahan JH, Brown JM (2014) IL-33 modulates chronic airway resistance changes induced by multi-walled carbon nanotubes. Inhal Toxicol 26:240–249. doi:10.3109/08958378.2014.880202
339. Wang P, Nie X, Wang Y, Li Y, Ge C, Zhang L, Wang L, Bai R, Chen Z, Zhao Y, Chen C (2013) Multiwall carbon nanotubes mediate macrophage activation and promote pulmonary fibrosis through TGF-beta/Smad signaling pathway. Small 9:3799–3811. doi:10.1002/smll.201300607
340. Mizutani N, Nabe T, Yoshino S (2012) Exposure to multiwalled carbon nanotubes and allergen promotes early- and late-phase increases in airway resistance in mice. Biol Pharm Bull 35:2133–2140, doi:DN/JST.JSTAGE/bpb/b12-00357 [pii]
341. Qu C, Wang L, He J, Tan J, Liu W, Zhang S, Zhang C, Wang Z, Jiao S, Liu S, Jiang G (2012) Carbon nanotubes provoke inflammation by inducing the pro-inflammatory genes IL-1beta and IL-6. Gene 493:9–12. doi:10.1016/j.gene.2011.11.046, S0378-1119(11)00716-5 [pii]
342. Hamilton RF Jr, Buford M, Xiang C, Wu N, Holian A (2012) NLRP3 inflammasome activation in murine alveolar macrophages and related lung pathology is associated with MWCNT nickel contamination. Inhal Toxicol 24:995–1008. doi:10.3109/08958378.2012.745633
343. Hamilton RF Jr, Xiang C, Li M, Ka I, Yang F, Ma D, Porter DW, Wu N, Holian A (2013) Purification and sidewall functionalization of multiwalled carbon nanotubes and resulting bioactivity in two macrophage models. Inhal Toxicol 25:199–210. doi:10.3109/08958378.2013.775197

344. Reisetter AC, Stebounova LV, Baltrusaitis J, Powers L, Gupta A, Grassian VH, Monick MM (2011) Induction of inflammasome-dependent pyroptosis by carbon black nanoparticles. J Biol Chem 286:21844–21852. doi:10.1074/jbc.M111.238519, M111.238519 [pii]
345. Xia T, Hamilton RF, Bonner JC, Crandall ED, Elder A, Fazlollahi F, Girtsman TA, Kim K, Mitra S, Ntim SA, Orr G, Tagmount M, Taylor AJ, Telesca D, Tolic A, Vulpe CD, Walker AJ, Wang X, Witzmann FA, Wu N, Xie Y, Zink JI, Nel A, Holian A (2013) Interlaboratory evaluation of in vitro cytotoxicity and inflammatory responses to engineered nanomaterials: the NIEHS Nano GO Consortium. Environ Health Perspect 121:683–690. doi:10.1289/ehp.1306561
346. Li R, Wang X, Ji Z, Sun B, Zhang H, Chang CH, Lin S, Meng H, Liao YP, Wang M, Li Z, Hwang AA, Song TB, Xu R, Yang Y, Zink JI, Nel AE, Xia T (2013) Surface charge and cellular processing of covalently functionalized multiwall carbon nanotubes determine pulmonary toxicity. ACS Nano 7:2352–2368. doi:10.1021/nn305567s
347. Yang M, Flavin K, Kopf I, Radics G, Hearnden CH, McManus GJ, Moran B, Villalta-Cerdas A, Echegoyen LA, Giordani S, Lavelle EC (2013) Functionalization of carbon nanoparticles modulates inflammatory cell recruitment and NLRP3 inflammasome activation. Small 9:4194–4206. doi:10.1002/smll.201300481
348. Ye S, Jiang Y, Zhang H, Wang Y, Wu Y, Hou Z, Zhang Q (2012) Multi-walled carbon nanotubes induce apoptosis in RAW 264.7 cell-derived osteoclasts through mitochondria-mediated death pathway. J Nanosci Nanotechnol 12:2101–2112
349. Chen T, Zang J, Wang H, Nie H, Wang X, Shen Z, Tang S, Yang J, Jia G (2012) Water-soluble taurine-functionalized multi-walled carbon nanotubes induce less damage to mitochondria of RAW 264.7 cells. J Nanosci Nanotechnol 12:8008–8016
350. Wang X, Guo J, Chen T, Nie H, Wang H, Zang J, Cui X, Jia G (2012) Multi-walled carbon nanotubes induce apoptosis via mitochondrial pathway and scavenger receptor. Toxicol In Vitro 26:799–806. doi:10.1016/j.tiv.2012.05.010, S0887-2333(12)00143-9 [pii]
351. Hamad I, Christy Hunter A, Rutt KJ, Liu Z, Dai H, Moein Moghimi S (2008) Complement activation by PEGylated single-walled carbon nanotubes is independent of C1q and alternative pathway turnover. Mol Immunol 45:3797–3803. doi:10.1016/j.molimm.2008.05.020, S0161-5890(08)00219-8 [pii]
352. Salvador-Morales C, Basiuk EV, Basiuk VA, Green ML, Sim RB (2008) Effects of covalent functionalization on the biocompatibility characteristics of multi-walled carbon nanotubes. J Nanosci Nanotechnol 8:2347–2356
353. Salvador-Morales C, Townsend P, Flahaut E, Venien-Bryan C, Vlandas A, Green MLH, Sim RB (2007) Binding of pulmonary surfactant proteins to carbon nanotubes; potential for damage to lung immune defense mechanisms. Carbon 45:607–617
354. Warheit DB, Donner EM (2010) Rationale of genotoxicity testing of nanomaterials: regulatory requirements and appropriateness of available OECD test guidelines. Nanotoxicology 4:409–413. doi:10.3109/17435390.2010.485704
355. Belyanskaya L, Manser P, Spohn P, Bruinink A, Wick P (2007) The reliability and limits of the MTT reduction assay for carbon nanotubes-cell interaction. Carbon 45:2643–2648
356. Casey A, Herzog E, Davoren M, Lyng FM, Byrne HJ, Chambers G (2007) Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity. Carbon 45:1425–1432
357. Worle-Knirsch JM, Pulskamp K, Krug HF (2006) Oops they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Lett 6:1261–1268
358. Monteiro-Riviere NA, Inman AO (2006) Challenges for assessing carbon nanomaterial toxicity to the skin. Carbon 44:1070–1078
359. Davoren M, Herzog E, Casey A, Cottineau B, Chambers G, Byrne HJ, Lyng FM (2007) In vitro toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. Toxicol In Vitro 21:438–448
360. Li X, Gao H, Uo M, Sato Y, Akasaka T, Abe S, Feng Q, Cui F, Watari F (2008) Maturation of osteoblast-like SaoS2 induced by carbon nanotubes. Biomed Mater 4:15005. doi:10.1088/1748-6041/4/1/015005, S1748-6041(09)85639-8 [pii]

361. Li X, Gao H, Uo M, Sato Y, Akasaka T, Feng Q, Cui F, Liu X, Watari F (2008) Effect of carbon nanotubes on cellular functions in vitro. J Biomed Mater Res A. doi:10.1002/jbm.a.32203
362. Cherukuri P, Gannon CJ, Leeuw TK, Schmidt HK, Smalley RE, Curley SA, Weisman RB (2006) Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence. Proc Natl Acad Sci U S A 103:18882–18886. doi:10.1073/pnas.0609265103
363. Chen J, Chen W, Zhu D (2008) Adsorption of nonionic aromatic compounds to single-walled carbon nanotubes: effects of aqueous solution chemistry. Environ Sci Tech 42:7225–7230. doi:10.1021/es801412j
364. Lin D, Xing B (2008) Adsorption of phenolic compounds by carbon nanotubes: role of aromaticity and substitution of hydroxyl groups. Environ Sci Tech 42:7254–7259. doi:10.1021/es801297u
365. Yang K, Zhu L, Xing B (2006) Adsorption of polycyclic aromatic hydrocarbons by carbon nanomaterials. Environ Sci Tech 40:1855–1861. doi:10.1021/es052208w
366. Yang K, Wu W, Jing Q, Zhu L (2008) Aqueous adsorption of aniline, phenol, and their substitutes by multi-walled carbon nanotubes. Environ Sci Tech 42:7931–7936. doi:10.1021/es801463v
367. Guo L, Von Dem Bussche A, Buechner M, Yan A, Kane AB, Hurt RH (2008) Adsorption of essential micronutrients by carbon nanotubes and the implications for nanotoxicity testing. Small 4:721–727. doi:10.1002/smll.200700754
368. Casey A, Davoren M, Herzog E, Lyng FM, Byrne HJ, Chambers G (2007) Probing the interaction of single walled carbon nanotubes within cell culture medium as a precursor to toxicity testing. Carbon 45:34–40
369. Casey A, Herzog E, Lyng FM, Byrne HJ, Chambers G, Davoren M (2008) Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells. Toxicol Lett 179:78–84. doi:10.1016/j.toxlet.2008.04.006, S0378-4274(08)00107-0 [pii]
370. Kapralov AA, Feng WH, Amoscato AA, Yanamala N, Balasubramanian K, Winnica DE, Kisin ER, Kotchey GP, Gou P, Sparvero LJ, Ray P, Mallampalli RK, Klein-Seetharaman J, Fadeel B, Star A, Shvedova AA, Kagan VE (2012) Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration. ACS Nano 6:4147–4156. doi:10.1021/nn300626q
371. Wang L, Castranova V, Mishra A, Chen B, Mercer RR, Schwegler-Berry D, Rojanasakul Y (2010) Dispersion of single-walled carbon nanotubes by a natural lung surfactant for pulmonary in vitro and in vivo toxicity studies. Part Fibre Toxicol 7:31. doi:10.1186/1743-8977-7-31, 1743-8977-7-31 [pii]
372. Fadel TR, Look M, Staffier PA, Haller GL, Pfefferle LD, Fahmy TM (2010) Clustering of stimuli on single-walled carbon nanotube bundles enhances cellular activation. Langmuir 26:5645–5654. doi:10.1021/la902068z
373. Li X, Gao H, Uo M, Sato Y, Akasaka T, Feng Q, Cui F, Liu X, Watari F (2009) Effect of carbon nanotubes on cellular functions in vitro. J Biomed Mater Res A 91:132–139. doi:10.1002/jbm.a.32203
374. Li X, Liu H, Niu X, Yu B, Fan Y, Feng Q, Cui FZ, Watari F (2012) The use of carbon nanotubes to induce osteogenic differentiation of human adipose-derived MSCs in vitro and ectopic bone formation in vivo. Biomaterials 33:4818–4827. doi:10.1016/j.biomaterials.2012.03.045, S0142-9612(12)00332-8 [pii]
375. Adamopoulos IE, Abstracts tSRMotACS, Little Rock, AR, United States, October 1–4 FIELD Full Journal Title:Abstracts, 64th Southwest Regional Meeting of the American Chemical Society, Little Rock, AR, United States, Duffin R, Mills Nicholas L, Donaldson K (2007) Nanoparticles-a thoracic toxicology perspective. Yonsei Med J 48:561–572
376. Liu X, Guo L, Morris D, Kane AB, Hurt RH (2008) Targeted removal of bioavailable metal as a detoxification strategy for carbon nanotubes. Carbon N Y 46:489–500
377. Kagan VE, Tyurina YY, Tyurin VA, Konduru NV, Potapovich AI, Osipov AN, Kisin ER, Schwegler-Berry D, Mercer R, Castranova V, Shvedova AA (2006) Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: role of iron. Toxicol Lett 165:88–100

378. Pensabene V, Vittorio O, Raffa V, Menciassi A, Dario P (2007) Investigation of CNTs interaction with fibroblast cells. In: Conference proceedings of IEEE engineering in medicine and biology society, vol 2007, pp. 6621–6624. doi:10.1109/IEMBS.2007.4353877
379. Gavello D, Fenoglio I, Fubini B, Cesano F, Premoselli F, Renna A, Carbone E, Carabelli V (2013) Inhibition of catecholamine secretion by iron-rich and iron-deprived multiwalled carbon nanotubes in chromaffin cells. Neurotoxicology 39:84–94. doi:10.1016/j.neuro.2013.08.008, S0161-813X(13)00132-0 [pii]
380. Meng L, Jiang A, Chen R, Li CZ, Wang L, Qu Y, Wang P, Zhao Y, Chen C (2013) Inhibitory effects of multiwall carbon nanotubes with high iron impurity on viability and neuronal differentiation in cultured PC12 cells. Toxicology 313:49–58. doi:10.1016/j.tox.2012.11.011, S0300-483X(12)00408-8 [pii]
381. Crouzier T, Nimmagadda A, Nollert MU, McFetridge PS (2008) Modification of single walled carbon nanotube surface chemistry to improve aqueous solubility and enhance cellular interactions. Langmuir 24:13173–13181. doi:10.1021/la801999n
382. Simon-Deckers A, Gouget B, Mayne-L'Hermite M, Herlin-Boime N, Reynaud C, Carriere M (2008) In vitro investigation of oxide toxicity and intracellular accumulation in A549 human pneumocytes. Toxicology 253:137–146
383. Jin H, Heller DA, Sharma R, Strano MS (2009) Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: single particle tracking and a generic uptake model for nanoparticles. ACS Nano 3:149–158
384. Nicola MD, Bellucci S, Traversa E, Bellis GD, Micciulla F, Ghibelli L (2008) Carbon nanotubes on Jurkat cells: effects on cell viability and plasma membrane potential. J Phys Condens Matter 20:474204
385. Sato Y, Yokoyama A, Shibata K, Akimoto Y, Ogino S, Nodasaka Y, Kohgo T, Tamura K, Akasaka T, Uo M, Motomiya K, Jeyadevan B, Ishiguro M, Hatakeyama R, Watari F, Tohji K (2005) Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1 in vitro and subcutaneous tissue of rats in vivo. Mol Biosyst 1:176–182
386. Tohji K, Sato Y (2006) Influence of purity and morphology on tissue reactivity of carbon nanotubes. Baiomateriaru 24:333–344
387. Simon-Deckers A, Gouget B, Mayne-L'hermite M, Herlin-Boime N, Reynaud C, Carriere M (2008) In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes. Toxicology 253:137–146
388. Wick P, Manser P, Limbach LK, Dettlaff-Weglikowska U, Krumeich F, Roth S, Stark WJ, Bruininck A (2007) The degree and kind of agglomeration affect carbon nanotube cytotoxicity. Toxicol Lett 168:121–131
389. Elgrabli D, Abella-Gallart S, Aguerre-Chariol O, Robidel F, Rogerieux F, Boczkowski J, Lacroix G (2007) Effect of BSA on carbon nanotube dispersion for in vivo and in vitro studies. Nanotoxicology 1:266–278
390. Moore VC, Leonard A, Price BK, Conyers JL, Tour JM (2007) The effect of physical characteristics on biocompatibility and cellular internalization of carbon nanotubes. In: 233rd ACS national meeting, Chicago, IL, United States, pp. INOR-531
391. Wu P, Chen X, Hu N, Tam UC, Blixt O, Zettl A, Bertozzi CR (2008) Biocompatible carbon nanotubes generated by functionalization with glycodendrimers. Angewandte Chemie 47:5022–5025
392. Fadel TR, Steenblock ER, Stern E, Li N, Wang X, Haller GL, Pfefferle LD, Fahmy TM (2008) Enhanced cellular activation with single walled carbon nanotube bundles presenting antibody stimuli. Nano Lett 8:2070–2076. doi:10.1021/nl080332i
393. Prencipe G, Tabakman SM, Welsher K, Liu Z, Goodwin AP, Zhang L, Henry J, Dai H (2009) PEG branched polymer for functionalization of nanomaterials with ultralong blood circulation. J Am Chem Soc 131(13):4783–4787
394. Li J, Xue Y, Han B, Li Q, Liu L, Xiao T, Li W (2008) Application of X-ray phase contrast imaging technique in detection of pulmonary lesions induced by multi-walled carbon nanotubes in rats. J Nanosci Nanotechnol 8:3357–3362

395. Al Faraj A, Cieslar K, Lacroix G, Gaillard S, Canet-Soulas E, Cremillieux Y (2009) In vivo imaging of carbon nanotube biodistribution using magnetic resonance imaging. Nano Lett 9(3):1023–1027
396. Lacerda L, Soundararajan A, Singh R, Pastorin G, Al-Jamal KT, Turton J, Frederik P, Herrero MA, Li S, Bao A, Emfietzoglou D, Mather S, Phillips WT, Prato M, Bianco A, Goins B, Kostarelos K (2008) Dynamic imaging of functionalized multi-walled carbon nanotube systemic circulation and urinary excretion. Adv Mater 20:225–230
397. Bussy C, Cambedouzou J, Lanone S, Leccia E, Heresanu V, Pinault M, Mayne-L'hermite M, Brun N, Mory C, Cotte M, Doucet J, Boczkowski J, Launois P (2008) Carbon nanotubes in macrophages: imaging and chemical analysis by X-ray fluorescence microscopy. Nano Lett 8:2659–2663. doi:10.1021/nl800914m
398. Jin H, Heller DA, Strano MS (2008) Single-particle tracking of endocytosis and exocytosis of single-walled carbon nanotubes in NIH-3T3 cells. Nano Lett 8:1577–1585
399. Liu Z, Li X, Tabakman SM, Jiang K, Fan S, Dai H (2008) Multiplexed multicolor Raman imaging of live cells with isotopically modified single walled carbon nanotubes. J Am Chem Soc 130:13540–13541
400. Herzog E, Casey A, Lyng FM, Chambers G, Byrne HJ, Davoren M (2007) A new approach to the toxicity testing of carbon-based nanomaterials-the clonogenic assay. Toxicol Lett 174:49–60
401. Zhou H, Mu Q, Gao N, Liu A, Xing Y, Gao S, Zhang Q, Qu G, Chen Y, Liu G, Zhang B, Yan B (2008) A nano-combinatorial library strategy for the discovery of nanotubes with reduced protein-binding, cytotoxicity, and immune response. Nano Lett 8:859–865

Index

P.V. Devarajan, S. Jain (eds.), *Targeted Drug Delivery: Concepts and Design*,
Advances in Delivery Science and Technology, DOI 10.1007/978-3-319-11355-5