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Nanoscience in Medicine Vol. 1

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Nanoscience in Medicine Vol. 1

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Preface

The arena of nanomedicine has developed rapidly due to numerous tailor-made nanomaterials and their ease of surface modification. In recent past, nanomaterials are reported to have noteworthy potential to manage diseases, and it is expected that they will change the face of medicine. Further, it has been realized that many of the vital concepts of nanomedicine have been overlooked, and they must be attended to utilize full potential of nanotechnology. Therefore, this book takes a systematic approach to address the gaps relating to nanomedicine and bring together fragmented knowledge on the advances on nanomaterials and their biomedical applicability. In particular, this book demonstrates an exclusive compilation of state of the art with a focus on fundamental concepts, current trends, limitations, and future directions of nanomedicine.

This book is also a platform to convey essential concepts of nanomedicine and how these concepts can be employed to develop advanced nanomaterials for a range of biomedical applications. Due to unique contribution of chapters from global leaders, this book has become an important reference source for scientists, teachers, doctors, research scholars, and university students, who are interested in the field. It also contains a textbook-like presentation of the important principles and applications of nanotechnology.

The first chapter by Meena has excellently introduced the nanovehicles for drug delivery system. The emerging nanocarriers for targeted drug delivery in cancer have been discussed in Chap. 2 by Singhvi and coauthors. In Chap. 3, Verma et al. have reviewed the therapeutic enzyme delivery mediated by gold nanoparticle. Improvement of vitamin A bioavailability by nanoencapsulation has been discussed by Maurya et al. in Chap. 4, and Rajak et al. have comprehensively discussed the nano-antimicrobials in Chap. 5. Chapter 6 is a critical discussion by Pramod on advanced oral delivery system for insulin using nanocarriers. The application of electrospun nanofibers for tissue engineering and regenerative medicine has been discussed by Johi et al. in Chap. 7. A multidimentional perspective on drug delivery system using solid lipid nanoparticles has been discussed by Pandey in Chap. 8. In Chap. 9 by Bansal et al., the overall perspective of nanomedicine in diagnosis and treatment has been discussed, while Chap. 10 by Nochehdehi et al. discusses the

iron- and cobalt-based bio-magnetic alloy nanoparticles for their biomedical applications. The application of nanoparticles in targeted and enhanced delivery of nucleic acid has been discussed in Chap. 11 by Penumarthi et al. Emam and coauthors have reviewed plasmonic hybrid nanocomposites for biomedical application in Chap. 12.

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About the Editors

Hemant Kumar Daima is an Assistant Professor at Amity University Rajasthan, India, and "Honorary Visiting Scientist" at RMIT University, Australia. He has over 10 years of research, teaching, and administrative experiences in various international organizations. Dr. Daima has significant expertise in designing nanoparticles with controlled physicochemical properties, and his research findings have revealed guiding principles involved in rational nanoparticle design approaches for biomedical applications. His research focuses on engineering the functional nanomaterials, controlling nano-bio interfacial interactions, and biomedical devices. He is Editorial Board Member and Reviewer of leading international publishers in the field of nanotechnology, nanotoxicology, and nanomedicine, with >39 peer-reviewed, high-impact publications to date. He has presented his research worldwide, and he is Member of several scientific/professional bodies. He is Recipient of numerous international fellowships/awards and has established Nano-Bio Interfacial Research Laboratory (NBIRL) to undertake high-quality fundamental and applied research. He obtained MSc (Biotechnology) from the University of Rajasthan, India, and PhD (Nanobiotechnology) from RMIT University, Australia.

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Nandita Dasgupta has completed her BTech and PhD from VIT University, Vellore, India, and is an Elected Fellow (FBSS) of Bose Science Society. She has major working experience in micro-/nanoscience and is currently working as Assistant Professor at the Department of Biotechnology, Institute of Engineering and Technology, Lucknow, India. Earlier at LV Prasad Eye Institute, Bhubaneswar, India, she has worked on mesenchymal stem cell-derived exosomes for the treatment of uveitis. She has exposure of working at university, research institutes, and industries including VIT University, Vellore, Tamil Nadu, India; CSIR-Central Food Technological Research Institute, Mysore, India; Uttar Pradesh Drugs and Pharmaceutical Co. Ltd., Lucknow, India; Indian Institute of Food Processing Technology (IIFPT), Thanjavur; and Ministry of Food Processing Industries, Government of India. At IIFPT, Thanjavur, she was involved in a project funded by a leading pharmaceutical company, Dr. Reddy's Laboratories, and has successfully engineered microvehicles for model drug molecules. Her areas of interest include micro-/nanomaterial fabrication and its applications in various fields – medicine, food, environment, and biomedical agriculture.

She has published 13 edited books and 1 authored book with Springer, Switzerland. She is an Associate Editor of *Environmental Chemistry Letters* – a Springer journal with an impact factor of 3.2.

Shivendu Ranjan has completed his BTech and PhD in Biotechnology from VIT University, Vellore, India, and has expertise in nano(bio)technology and is an Elected Fellow of Bose Scientific Society (FBSS). He is currently working as Head, Research & Technology Development at E-Spin Nanotech Pvt. Ltd., SIDBI Center, Indian Institute of Technology, Kanpur, India. After joining E-Spin Nanotech, IIT Kanpur, he has successfully developed prototypes for many products and three patents. He is also serving as a Senior Research Associate (Adjunct) at Faculty of Engineering & Built Environment, University of Johannesburg, Johannesburg, South Africa. He is also mentoring Atal Innovation Centre, Bhubaneswar, Odisha, giving his technical inputs to the centre. Atal Innovation Centre is the part of Atal Innovation Mission of the NITI Aayog, Govt of India. He is also Reviewer of Iran National Science Foundation (INSF), Tehran, Iran, and Jury at Venture Cup, Denmark. He had founded and drafted the concept for the first edition of the "VIT Bio Summit" in 2012, and the same has been continued till date by the university. He has worked in CSIR-CFTRI, Mysuru, India, as well as UP Drugs and Pharmaceutical Co. Ltd., India, and IIFPT, Thanjavur, MoFPI, Government of India. At IIFPT, Thanjavur, he was involved in a project funded by a leading pharmaceutical company, Dr. Reddy's Laboratories, and has successfully engineered micro-vehicles for model drug molecules.

His research interests are multidisciplinary and include: micro-/nanobiotechnology, nano-toxicology, environmental nanotechnology, nanomedicine, and nanoemulsions. He is an Associate Editor of *Environmental Chemistry Letters* – a Springer journal. He has published six edited books and one authored book with Springer, Switzerland. He has published many scientific articles in international peer-reviewed journals and has authored many book chapters as well as review articles. He has received several awards and recognitions from different national and international organizations.

Eric Lichtfouse PhD, born in 1960, is an Environmental Chemist working at the University of Aix-Marseille, France. He has invented carbon-13 dating, a method allowing to measure the relative age and turnover of molecular organic compounds occurring in different temporal pools of any complex media. He is teaching scientific writing and communication, and has published the book *Scientific Writing for Impact Factors*, which includes a new tool – the micro-article – to identify the novelty of research results. He is Founder and Chief Editor of scientific journals and series in environmental chemistry and agriculture. He founded the European Association of Chemistry and the Environment. He received the Analytical Chemistry Prize by the French Chemical Society, the Grand Prize of the Universities of Nancy and Metz, and a Journal Citation Award by the Essential Indicators.

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Chapter 1
Nanomaterials: A Promising Tool for Drug Delivery

Priyanka Kumari, Suaib Luqman, and Abha Meena

Contents

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Abstract Nanotechnology is an ingenious approach that has potential utilization in the drug delivery system. Presently, many of the natural or synthetic nanomaterials are under investigation for their potential to be used as drug delivery tools. Nanomaterials have also shown potential impeding interest in overcoming new/existing drug problems like low solubility, bioavailability, target specificity, toxicity, stability, side effects, and early-stage degradation. In this chapter, we have discussed how nanomaterials act as a prospective tool in overcoming such precincts and their role in different therapies and drug delivery approaches. In addition, a list of the nanomaterials which are or could be used as a drug delivery tool is also mentioned along with the selected success stories besides certain limitations. In the end, the challenges faced by nanomaterials in biomedical science have also been pointed out.

Keywords Drug carrier · Nanomaterial · Cancer therapy · Drug delivery

1.1 Introduction

According to the US National Nanotech Initiative, "Nanotechnology is the understanding and control of matter at dimensions between 1 and 100 nm, where unique phenomena enable novel applications". The size range which is offered by nanotechnology is one thousand millionth of a particular unit, i.e., 1 nm = 10^{-9} m. Nanotechnology deals with the mechanism that appeared at the nanoscale and molecular level. It can also be defined as the manipulation of matter on an atomic and molecular scale which includes layout, production, characterization, and application in different fields, mainly in the area of medicine, also known as nanomedicine and medical science (Martin 2006). The challenges faced by conventional drug feels a necessity for the development of such nanomaterials and nanomedicine, which can be used in the treatment and/or diagnosis of diseases. This leads to the emergence of nanoparticles in the field of pharmaceutical sciences. The combination of the nanoparticles with advanced techniques in spectroscopy and optics has led to the advancement in relieving pain, treating traumatic injury, preventing disease, and therefore improving human health issues (Huang et al. 2017). The broad spectrum of nanoscale techniques led to the advancement in the field of medical science in terms of disease, diagnosis, prevention and treatments (Singh and Lillard 2009). The application of nanotechnology in medicine and more specifically drug delivery is spreading its web rapidly (De Jong and Borm 2008). In the recent past, many anticancer molecules, phytochemicals, and synthetic drug have been discovered, which are under exploration for drug delivery purpose. It has revealed a promising potential in maintaining health along with preventing and treating diseases. However, the efficacy of these drugs is mainly dependent on their delivery at a controlled rate. Many drugs, instead of showing therapeutic benefits, face solubility issues. Such limitation proves that the drug is a poor candidate for therapeutic usages and has also been identified as a key barrier in the successful development

and clinical use of anticancer molecules (Narvekar et al. 2014). About more than 40% of the drug available in the market have poor water solubility, in addition to the low absorption, and almost 70% of new drug entities are found to have poor solubility (Kawabata et al. 2011; Savjani et al. 2012). In the area of drug research and development, most of the complications arise from the poor aqueous solubility of drug entities. The poor solubility of a drug limits its dissolution rate which results in its low bioavailability (Kawabata et al. 2011). Cancer is among the foremost diseases responsible for the higher death rates in developing and developed countries. Various drugs with effective therapeutic activities are available for the treatment of cancer. However, because of certain limitations, these drugs are not fully effective during treatment. For example, camptothecin is known to show high antitumor activity; still, its application in the form of a drug is limited because of its stability, poor solubility, and side effect problems (Du et al. 2014). The nontargeting nature of the drug is also one of the major issues in achieving optimum drug delivery. The drug named milrinone, which is known as a cardiac inotrope and vasodilator, is commercially available in the form of lactate formulation. However, due to its nontarget specificity, low bioavailability, and side effects of palpitations and renal dysfunction, its potency may be limited (Lomis et al. 2017). Doxorubicin is also the most commonly known antibiotic drug, which is used to fight against tumors and leukemia. However, due to its less potency against cancer and toxic side effects, its therapeutic use is limited (Haghiralsadat et al. 2017). Many bacterial species are found to be resistant to antibiotics and antimicrobial drugs which limit their permeability to the cell membrane (Nicolosi et al. 2015). The reduced cell membrane permeability has been investigated as a major issue which leads to the spread of diseases like malaria, tuberculosis, and typhoid fever. The drug primaquine is the only drug which is in clinical use. It antagonizes the worsening condition of malaria caused by *Plasmodium vivax* and *Plasmodium ovale.* Hence used for the radical cure of the relapsing malaria (Baird and Hoffman 2004; Gathirwa et al. 2014). However, a higher dose of primaquine can produce toxic side effect, leading to gastrointestinal and hematological problems, and it also faces bioavailability issues (Gathirwa et al. 2014). Curcumin is known for its antioxidant, anti-inflammatory, antitumor, anti-HIV, and antimicrobial activities with no toxic effects (Bhawana et al. 2011). Many reports suggest that curcumin also faces problems of water insolubility at physiological pH, poor bioavailability, high metabolism, and elimination (Zhang et al. 2015). The enhancement of the therapeutic effects of potential molecules is very important for clinical use by reducing issues like toxicity, bioavailability, and solubility. The development of nanotechnology approaches provides an opportunity to overcome issues associated with existing drug and/or potential drug molecules. The development of nanotechnological approaches provides an opportunity to eradicate issues related to prospective molecules by improving their solubility, bioavailability, and stability to increase their half-lives. These approaches are well-known for the targeted delivery of drug molecules by eliminating side effects (De Jong and Borm 2008). So far, many polymeric nanoparticles and nanotechnology products are approved by the US Food and Drug Administration (USFDA) for clinical use, and various others are under clinical and preclinical progress which

may increase in the future due to its advantages (Davis et al. 2010a, b). Another important factor which affects the behavior of nanomaterials is its biodegradability. The selected examples of nanomedicines which are undergoing clinical trials and/or USFDA approved are mentioned in Table 1.1.

Several natural and synthetic nanoparticles are also under consideration for their use in drug delivery owing to the advantage of surface alteration and stability by which controlled release and specific site localization in inflamed tissues can be achieved. They are known to play a major role in targeted drug delivery without any side effects, enhancing bioavailability and eradicating the drug solubility problem for systemic delivery (Singh and Lillard 2009). Not only advantages, but nanomaterials also have certain disadvantages as listed in Table 1.2 (Gratieri et al. 2010; Ds et al. 2016). Nanoparticles can also deliver drugs to the brain by crossing the blood-brain barrier. Nano-sizing of the targeted delivery systems also enhances the drug dissolution rate, reduces toxicity, increases patient compliance, increases the surface area, and minimizes the dose requirement. The cell and tissue distributions are modified by drug-loaded nanoparticles which led to the delivery of selected compounds to increase the efficacy of the drug and eliminate drug toxicity issues (Fakruddin et al. 2012). Nanotechnology-based approaches enhance the efficacy as well as eliminate the side effect profile associated with the drug. Considering these unique properties of drug delivery systems, nanoparticles have exhibited a promising role in delivering diverse potent molecules to the site of their action in the body exhibiting a targeted effect (Singh and Lillard 2009). These advantages may also lead to the evolution of successful potential nanomaterials which can transform the pharmaceutical industry.

1.2 Characteristics of Nanomaterial Critical for Drug Delivery

The physiochemical properties of nanomaterials are key characteristics which influence their in vivo distribution and behavior. Hence, this section describes the basic properties associated with nanomaterial with a focus on drug delivery aspect.

1.2.1 Particle Size and Shape

The particle size and shape of nanomaterials have a vital role in determining their behavior, biodistribution under in vivo conditions, biological fate, toxicity, and targeting capability (Powers et al. 2007). In addition, it also influences the loading capacity, release profile, and stability of nanomaterials. It has been observed that the particle size of 200 nm or more tends to stimulate the lymphatic system and is quickly eliminated from the circulation (Prokop and Davidson 2008). It has been

Table 1.1 List of selected examples of nanomedicines which are undergoing clinical trials and USFDA approval

Sr. no.	Name	Material description	Active ingredient	Application	Company	References
1	Adagen/ pegademase bovine	PEGylated adeosine deaminase enzyme	Pegademase bovine	Severe combined immunodeficiency disease	Sigma-Tau Pharmaceuticals	Bobo et al. (2016)
2	DepoCyt©	Liposomes	Cytarabine	Lymphomatous meningitis	Sigma-Tau Pharmaceuticals	Bobo et al. (2016)
3	Doxil®/ Caelyx™	PEGylated liposomes	Doxorubicin	Kaposi's sarcoma, ovarian cancer, multiple myeloma	Janssen Pharmaceutica	Bobo et al. (2016)
4	Myocet	Liposomes	Doxorubicin hydrochloride	Metastatic breast cancer	Elan Pharmaceuticals/ Sopherion Therapeutics	Wang et al. (2015)
5	DaunoXome	Liposomes	Daunorubicin	HIV-related Kaposi's sarcoma	Gilead Sciences	Pillai (2014)
6	Marqibo®	Liposomes	Vincristine	Acute lymphoblastic leukemia	Onco TCS	Bobo et al. (2016)
7	Onivyde®	Liposomes	Irinotecan	Pancreatic cancer	Merrimack Pharmaceuticals	Bobo et al. (2016)
8	Onco-TCS	Liposomes	Vincristine	Non-Hodgkin lymphoma	Inex Pharmaceuticals	Bulbake et al. (2017)
9	Aroplatin	Liposomes	Cisplatin analog	Colorectal cancer	Antigenics, Inc.	Bulbake et al. (2017)
10	OSI-211	Liposomes	Lurtotecan	Lung cancer/recurrent ovarian cancer	OSI Pharmaceuticals	Bulbake et al. (2017)
11	Amphotec®	Liposomes	Amphotericin B	Severe fungal infections	Ben Venue Laboratories Inc.	Bulbake et al. (2017)
12	Visudyne®	Liposomes	Verteporfin	Choroidal neovascularization	Novartis	Bulbake et al. (2017)
13	DepoDur™	Liposomes	Morphine sulfate	Pain management	SkyPharma Inc.	Bulbake et al. (2017)
14	Epaxal®	Liposomes	Inactivated hepatitis A virus	Hepatitis A	Crucell, Berna Biotech	Bulbake et al. (2017)

(continued)

Table 1.1 (continued)

Sr. no.	Name	Material description	Active ingredient	Application	Company	References
15	Exparel®	Liposomes	Bupivacaine	Pain management	Pacira Pharmaceuticals, Inc.	Bulbake et al. (2017)
16	Mepact® (2004)	Liposomes	Mifamurtide	High-grade, resectable, nonmetastatic osteosarcoma	Takeda Pharmaceutical Limited	Bulbake et al. (2017)
17	ThermoDox	Liposomes	Doxorubicin	Hepatocellular carcinoma	Celsion Corporation	Pillai (2014)
18	Atragen	Liposomes	trans retinoic acid	Acute promyelocytic leukemia	Oasmia	Pillai (2014)
19	Paclical	Polymeric micelle	Paclitaxel	Ovarian cancer	Pharmaceutical AB	Pillai (2014)
20	Genexol-PM	PEG-poly(D, L-lactide)	Paclitaxel	Breast cancer/small cell lung cancer	Samyang	Pillai (2014)
21	Narekt-102	PEGylated liposomes	Irinotecan	Breast cancer/colorectal cancer	Nektar Therapeutics	Pillai (2014)
22	NKTR-105	PEG	Docetaxel	Solid tumors	Nektar Therapeutics	Pillai (2014)
23	Eligard®	Poly(D,L-lactide-co-glycolide)	Leuprolide acetate	Prostate cancer	Tolmar	Bobo et al. (2016)
24	Macugen®/ pegaptanib	Polyethylene glycol	Anti-VEGF aptamer	Macular degeneration, neovascular age-related	Bausch & Lomb	Bobo et al. (2016)
25	Estrasorb™	Micelles	Estradiol	Menopausal therapy	Novavax	Bobo et al. (2016)
26	Avinza®	Nanocrystals	Morphine sulfate	Psychostimulant	Pfizer	Bobo et al. (2016)
27	Copaxone®	L-Glutamic acid, L-alanine, L-lysine, and L-tyrosine	Glatiramer acetate	Multiple sclerosis	Teva	Bobo et al. (2016)
28	Abraxane®	Albumin	Paclitaxel	Breast cancer, NSCLC, pancreatic cancer	Celgene	Bobo et al. (2016)
29	DepoDur®	Liposomes	Morphine sulfate	For treatment of chronic pain in patients requiring a long-term daily around-the-clock opioid analgesic (administered into the epidural space)	SkyePharma PLC	Weissig et al. (2014)
31	CRLX101	Cyclodextrin	Camptothecin	Various cancers	Cerulean Pharma	Wang et al. (2013)

32	NC-6004	PEG-polyaspartate	Cisplatin	Various cancers	NanoCarrier Co.	Wang et al. (2013)
33	NK-105	PEG-polyaspartate	Paclitaxel	Various cancers	Nippon Kayaku Co. Ltd.	Wang et al. (2013)
34	Genexol-PM	PEG-poly(D, L-lactide)	Paclitaxel	Non-small cell lung cancer	Samyang Biopharmaceuticals	Ulbrich et al. (2016)
35	Genexol-PM	PEG-poly(D, L-lactide)	Paclitaxel	Recurrent or metastatic breast cancer	Samyang Biopharmaceuticals	Ulbrich et al. (2016)
36	Genexol-PM	PEG-poly(D, L-lactide)	Paclitaxel	Pancreatic cancer bladder cancer, ureter cancer	Samyang Biopharmaceuticals	Ulbrich et al. (2016)
37	Lipotecan	Polymeric micelle	TLC388 (CPT derivate)	Advanced solid tumor, advanced/ metastatic RCC patients	Taiwan Liposome Co. Ltd.	Ulbrich et al. (2016)
38	Nanoxel	mPEG-poly (D,L-lactic acid)	Paclitaxel	Advanced breast cancer	Fresenius Kabi	Ulbrich et al. (2016)
39	NC-4016	PEG-b-poly(L-glutamic acid)	Oxaliplatin	Advanced cancers lymphoma	NanoCarrier Co.	Ulbrich et al. (2016)
40	NC-6004 (Nanoplatin)	mPEG-b-poly(L-glutamic acid)	Cisplatin	Pancreatic cancer	NanoCarrier Co.	Ulbrich et al. (2016)
41	NK-911	PEG-b-poly(α,β-aspartic acid)	Doxorubicin	Various solid tumors	Nippon Kayaku	Ulbrich et al. (2016)
42	NK-012	PEG-b-poly(L-glutamic acid)	SN-38	Refractory solid tumors advanced solid tumors, metastatic colorectal cancer	Nippon Kayaku	Ulbrich et al. (2016)
43	NK-012	PEG-b-poly(L-glutamic acid)	SN-38	Solid tumors, small cell lung cancer, breast cancer	Nippon Kayaku	Ulbrich et al. (2016)
44	NC-6300	PEG-b-poly(α,β-aspartatehydrazone)	Epirubicin	Solid tumors	NanoCarrier Co.	Ulbrich et al. (2016)
45	SP1049C	Pluronic micelles	Doxorubicin	Gastric cancer	Supratek Pharma	Singh and Lillard (2009)

(continued)

Table 1.1 (continued)

Sr. no.	Name	Material description	Active ingredient	Application	Company	References
46	Xyotax®	Polymer-drug conjugates	Paclitaxel	Primary peritoneal carcinoma, recurrent ovarian carcinoma	Gynecologic Oncology Group	Banik et al. (2016)
47	PK1	Polymer-drug conjugates	Doxorubicin	Breast cancer	University of Glasgow	Banik et al. (2016)
48	Annamycin	Liposomes	L-Annamycin	Acute lymphocytic leukemia	Callisto Pharmaceuticals	Banik et al. (2016)
49	Intelence®	Hydroxylpropyl methylcellulose	Etravirine	Treatment of HIV	Janssen Therapeutics, USA	Kalepu and Nekkanti (2015)
50	Kaletra®	PVP/VA	Lopinavir/ritonavir	Treatment of HIV	Abbott Laboratories, USA	Kalepu and Nekkanti (2015)
51	Fenoglide®	PEG/poloxamer	Fenofibrate	To reduce cholesterol levels in people at risk of cardiovascular disease	Santarus, Inc.	Kalepu and Nekkanti (2015)
52	DO/NDR/02	Polymeric micelles	Paclitaxel	Breast cancer	Dabur Research Foundation	Kalepu and Nekkanti (2015)
53	Flucide	Polymeric micelles	Anti-influenza	Used for fungal infection	NanoViricides	Kalepu and Nekkanti (2015)

Table 1.2 Some risks and benefits of nanomaterial drug delivery system

Sr. no.	Risks	Benefits
1.	The occurrence of inflammation and fibrosis due to phagolysosomal membrane permeability and generation of reactive oxygen species	Controlled and sustained release of the drug at the site of localization, cellular uptake and frequent elimination of the drug for better therapeutic efficacy
2.	Small size could have adverse effects, as exposure to cellular components becomes higher	Better utilization of the hydrophobic as well as hydrophilic drug molecule
3.	The risk in development and sterilization of nanomaterials, since chemical agents can enhance cytotoxicity and poor stability	Direct and selective targeting of the therapeutics to cancer cells (both passive and active targeting)
4.	The aggregation state of the nanomaterials could be a potential risk	Targeted drug delivery using nanomaterials is an effective approach. It is less costly and has low toxicity on healthy cells
5.	Burst effect and limited drug loading may cause intolerability if $\geq$10 μm	Some nanomaterials are biocompatible and biodegradable

confirmed that different biological mechanisms like cellular uptake, endocytosis, and capability of the particle in the endocytic route generally depend on the particle size of the nanomaterial (Aillon et al. 2009). The particle size, distribution, and shape of nanomaterials can be studied using various analytical techniques like photon-correlation spectroscopy (PCS), dynamic light scattering (DLS) or quasi-elastic light scattering (QELS), dark-field microscopy, acoustic spectrometry measurement, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) (Lim et al. 2013b). It has been reported that the size of nanomaterials also predicts their pharmacological behavior. It has been shown that nanomaterials less than 50 nm size rapidly crossed nearly all the tissues and showed toxic demonstration in various tissues, whereas nanomaterials more than 50 nm (in particular 100–200 nm positively charged particles) are taken up by the reticuloendothelial system which restricts their passage to other tissues (De Jong et al. 2008). The elimination of nanomaterials takes place in the reticuloendothelial system, which protects other tissues and makes the reticuloendothelial system as the main target of oxidative stress. Many studies have shown that smaller-dimension nanomaterials, i.e., less than 100 nm, lead to adverse respiratory effects, as compared to larger-dimension nanomaterials (Gurr et al. 2005). In a few specific cases, the in vivo experiment demonstrated that particle size in the range of 150 nm and negatively charged can enter through the tumor tissues. Studies also suggest that particle size of 50–100 nm having a slightly positive charge can enter through large tumors. Hence, nanoparticles in the range of 10–100 nm size with surface charge might enter through tumors when administered into the circulatory system (Davis et al. 2010a, b). The particle size also influences the oral toxicity profile of nanomaterials. Oral toxicity generally increases with decreases in particle size. It was observed that the oral toxicity of copper nanoparticles gets enhanced with a decrease in size. So, it was noted that larger-sized particles were nontoxic even at a

higher dose, and smaller-sized particles were found to be somewhat toxic (Chen et al. 2006). The shape of the nanomaterial affects the compatibility with the biological system and retention period in tissues and organs. The performance of the nanomaterial can be improved by controlling the shape of the nanomaterial (Lin et al. 2014). In one study, it has been shown that plate-shaped silver nanoparticles were dangerous than a rod- or wirelike structure when tested against zebrafish embryos and *Escherichia coli* (George et al. 2012). It has been demonstrated that endocytosis of spherically shaped nanomaterials is facile, less toxic, and rapid compared to rod-shaped or fiber-like nanostructures, whether they are homogenous or heterogeneous (Lee et al. 2007). Non-spherical nanostructured materials are more readily responsible for other biological issues (Kim et al. 2012). Rod-shaped carbon nanotubes were found to block K^+ ion channels more effectively, compared to spherical carbon fullerenes, as evaluated in several studies (Park et al. 2003). It has been found that TiO_2 fibers are more cytotoxic than the spherically shaped nanomaterials (Hsiao and Huang 2011).

1.2.2 Surface Properties

In the context of drug delivery, the surface properties of nanomaterials have been considered to be crucial for the environment of the biological fluid system. Among the different surface properties of nanoparticles, its composition, energy, charge, absorbance, and adhesion to the surface are considered to be essential elements. The composition of the nanomaterial surface is associated with the one-dimensional layer of the surface and usually estimated by energy-dispersive X-ray spectroscopy (EDX) and other related compositional analysis. The surface energy of the nanomaterial is correlated with aggregation, dissolution, and accumulation. The surface charge shows the stability and aggregation properties of the nanomaterial and is generally predicted by zeta potential (Lin et al. 2014). The zeta potential measures the surface charge of the nanomaterials. The zeta potential with a value of ±30 mV is typically preferred to evaluate the stability of nanomaterials. A value greater than 30 mV indicates a stable condition, whereas a value less than 30 mV indicates a condition toward aggregation, instability, coagulation, or flocculation (Sapsford et al. 2011). The decrement in the particle size of nanomaterials leads to increment in the surface area relative to volume, making the surface of the nanomaterial more reactive to itself and the surrounding environment (Powers et al. 2007). This signifies that most of the drug is closer to the surface of the nanomaterial which leads to faster drug release (Buzea et al. 2007). However, modification of the surface properties is another possibility to produce ideal nanomaterials. Nanomaterials can easily be identified by the lymphatic system, exposed to the body's immune response as foreign particles. When these nanoparticles enter the bloodstream, unmodified surface nanomaterials are opsonized at a very fast rate and cleared through the mononuclear phagocyte system. To achieve successful drug targeting, it is very

crucial to reduce the chances of opsonization and increase the retention period of nanoparticles in the cellular system. So, elimination of the nanomaterials must be addressed. As the nonmaterial is more hydrophobic, it is more likely to be cleared easily due to enhanced binding of components of blood; it seems essential to make them hydrophilic to enhance their circulation period in the biological system.

These issues may be addressed by coating a nanomaterial with different natural/synthetic polymers with hydrophilic properties, e.g., polyethylene glycol (PEG), polyethylene oxide (PEO), and polysorbate 80 (Tween 80), which has been proven valuable (Araujo et al. 1999; Rizvi and Saleh 2018). Few reports revealed that coating of polyethylene glycol on the surface of the nanomaterial alters opsonization and prevents frequent drug loss. PEGylated nanomaterials (also referred to as "stealth" nanoparticles) remain unexposed to the reticuloendothelial system (Li and Huang 2010). The clearance of nanomaterials can be addressed by creating polymer complexes, but the problem of aggregation is still a concern. There are several nanoparticles, like micelles, quantum dots and dendrimers, which are susceptible to aggregation. Many approaches have been selected to prevent these issues (Li and Kaner 2005).

1.2.3 Drug Loading and Release

The drug loading capacity of a nanomaterial is defined as the quantity of drug bound per mass of nanoparticle, or in other words it is the moles of drug per mg nanoparticle or mg drug per mg nanoparticle. For successful targeted delivery, the nanomaterial should have higher drug loading capacity. Usually, drug loading is accomplished by two methods. In the first method, the drug is mixed at the time of preparation of nanoparticle, and in the second method, absorption of the drug is done afterward. It is attained by incubating the nanoparticle with the solution of the drug. Drug loading and entrapment efficiencies are significant parameters, which specifically depend on the solubility of the drug with the material of the nanoparticle, molecular weight, interactions between drug and the nanomaterial, and any existence of the functional group in the drug or nanomaterial. According to some reports, polyethylene glycol (PEG) has fewer problems or no problem with drug loading. Studies also showed that the use of ionic bonding between drug and nanomaterial could be effective in enhancing the loading capacity of the drug (Singh and Lillard 2009). Drug releasing from nanomaterials is considered to be a very important parameter in drug delivery. The foremost target for controlled drug release is to maintain the drug concentration in the blood within the therapeutic range. Hence, it is optimal to produce a drug carrier system that sustains low dosing and contributes in controlled release (Siegel and Rathbone 2012). The profile of drug releasing is mainly dependent on pH, temperature, solubility, diffusion of drug through a matrix of the nanomaterial, degradation of the nanomaterial, and adsorption of the drug. If the diffusion rate of the drug is higher than the matrix of the nanomaterial, then the releasing profile is greatly affected by the diffusion process. The releasing profile is

also dependent on the method of incorporating the drug to the nanomaterial (Son et al. 2017). There are various methods that can be used to study the release of drug from the nanoparticle such as diffusion through cells with artificial or biological membranes, dialysis bag diffusion method, reverse dialysis bag diffusion method, stirring followed by ultracentrifugation/centrifugation, or ultrafiltration. To save time and technical issues in separating nanoparticles from release media, the dialysis method is commonly used. However, these methods are hard to duplicate and scale up for industrial purposes (Singh and Lillard 2009). The release pattern of the drug will always vary depending upon the type of nanomaterial used. The drug release from a nanomaterial is mainly affected by different parameters which include the composition of the nanomaterial matrices, physical and chemical interaction among constituents, the ratio of the chemical constituents, and the manufacturing procedures (Langer and Peppas 1983; Siegel and Rathbone 2012). One example of the drug-releasing profile of nanomaterials is curcumin-encapsulated solid lipid nanomaterial. Curcumin is a low-molecular-weight plant-based drug with a broad spectrum of activities like anti-inflammatory, antibacterial, anticarcinogenic, anti-tumorigenic, anti-ischemic, anticoagulant, and wound healing (Shaikh et al. 2009). However, it still faces several issues like poor bioavailability, low gastrointestinal absorption, poor solubility, and fast degradation. Curcumin-encapsulated solid lipid nanoparticles showed greater than 85% and 92% of curcumin release after 36 and 48 h, respectively (Jourghanian et al. 2016).

1.2.4 Interaction of Nanomaterial with a Biological System and Targeted Drug Delivery

As nanomaterials enter into the human body, many disagreeable effects like aggregation and coagulation might occur within the system. It could be due to various intermolecular interactions between biomolecules and the interface of nanomaterials or by surrounding mediating fluids. The surface features of the nanomaterial are characterized by the physical and chemical properties like chemical ingredients, crystallinity, and geometry of the surface, shape, porosity, dissolution, surface charge, size distribution, agglomeration, aggregation, and dispersion stability of the nanomaterial. Along with this, important properties of the biological environment include pH, ionic strength, polarity, viscosity, surface tension, temperature, etc. The convenient physiochemical characterization of the nanomaterials should be generalized depending upon the physical states of nanomaterials (Nel et al. 2009). After identifying the influence of the nanomaterial modifications for successful drug delivery, the next stage is the production of targeted drug delivery. Nanomaterials can enter the inflamed or injured tissue due to bigger epithelial assemblage. This insertion is through active or passive targeting mechanisms (Fig. 1.1). There have been various reports regarding the development of nanomaterial as drug delivery systems for cell-specific targeting by applying passive and active targeting mechanisms

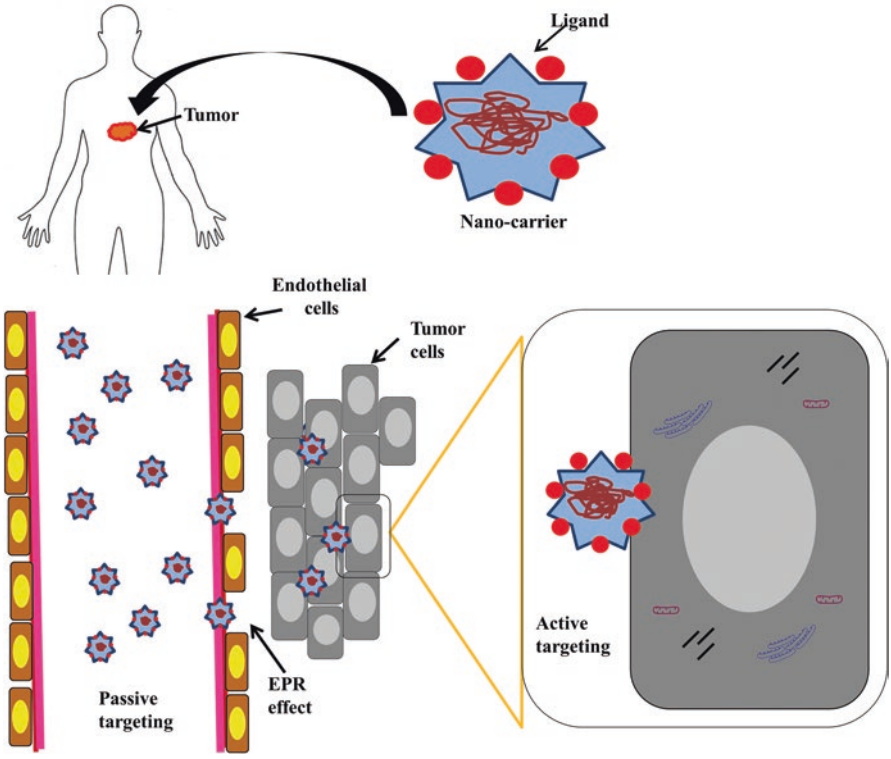

Fig. 1.1 Schematic representation of the passive and active targeting mechanism of drug delivery

(Varshosaz and Farzan 2015). Passive targeting takes place due to pathophysiological properties of the tumor vessels like extravasation of the vascular system and poor lymphatic drainage. Passive targeting is carried out by combining the therapeutic agent with nanoparticles that passively reach the targeted site of action. Several substances like polyethylene glycol (PEG), a hydrophilic agent, when added to the surface of the nanomaterial, allow the water molecules to bind to the oxygen molecules present on the surface of PEG through hydrogen bonding. The nanomaterial surrounded by the hydration film, attaining an anti-phagocytic property, due to hydrophobic interactions that are normal to the reticuloendothelial system. Thus, the nanomaterials can stay for a prolonged duration in the circulation system, without any wastage of drug (van Vlerken et al. 2007). The entrapped drug in the nanomaterial may passively target tumors through enhanced permeability and retention effect. Genexol-PM®, a poly(D,L-lactide) polymeric micelle formulation, is one of the examples of passive targeting nanomedicine, which is responsible for the controlled release of the drug (approved in Korea in 2007).

About 90% of therapeutic agents will surely get loaded into the reticuloendothelial organs like the liver and spleen because of elimination through mononuclear

phagocytes. Active targeting is being examined by attaching the therapeutic agent-loaded nanocarrier to the target site through specific interactions to achieve spatial localization to diseased sites, without any off-targeting (Albanese et al. 2012). These interactions comprise of ligand-receptor binding and antigen-antibody reaction. The decoration of the surface of the nanomaterial with the ligand can promote binding to the specific target cells and activate target-mediated endocytosis (Zhang et al. 2012). CALAA-01, a cyclodextrin-containing cationic polymer, polyethylene glycol corona, and human transferrin as a ligand, is the first targeted nanomaterial delivery formulation to feature siRNA. The transferrin present on the nanomaterial surface attached to transferrin receptors exists on cancer cells, and the nanomaterials enter through receptor-mediated endocytosis. The siRNA encapsulated nanomaterials are introduced to melanoma patients intravenously. They are distributed in the body and finally get localized in tumors (Davis et al. 2010a, b).

1.2.5 Biodegradability

The biodegradability behavior of the nanomaterials plays an important role in their applications in health care. The uniqueness of the nanomaterials encourages their application, but it also enhances their chance to cause a destructive effect to an organism which is unfamiliar to their unique properties (Maynard 2014). It is confirmed that solid, non-biodegradable nanomaterials generally cause the formation of reactive oxygen species (ROS) and beginning of autophagy, but the mechanism is still unknown (Logan et al. 2014; Cohignac et al. 2014; Chiu et al. 2015). The formation of ROS was observed in different cell lines that were incubated with tough, non-biodegradable spherical nanomaterials (Yu et al. 2014; Guo et al. 2015). It was reported that titanium oxide nanorods led to the generation of autophagosome-like vacuoles in bronchial epithelial cells, followed by apoptosis (Park et al. 2014a). In one study, it was found that mouse RAW264.7 macrophages, when exposed to concentration-dependent SWCNTs (~1 to 7 μg/ml), displayed 50% increment in ROS (Park et al. 2014b). These concentrations are found less than the quantity needed for ROS formation when 60-nm spherical-shaped silica nanoparticles were added to HepG2 cells (Yu et al. 2014). Based on certain limitations regarding non-biodegradability, researchers mainly focused on biodegradable nanomaterials. Biodegradable nanomaterials can be easily prepared from a variety of materials like polysaccharides, proteins, and certain synthetic biodegradable polymers. Biodegradable nanomaterials have been used for the site-specific delivery of vaccines (Gutjahr et al. 2016), drugs, and several other biomolecules (Kumari et al. 2010; Chan et al. 2010). Several biodegradable nanomaterials which are frequently used for the preparation of nanomaterials are polylactic acid (PLA), poly-ε-caprolactone (PCL), poly-D,L-lactide-co-glycolide (PLGA), cellulose, chitosan, etc. The positive effect of doxorubicin incorporated in poly(isohexylcyanoacrylate) was observed in mice. It showed a proper distribution of the drug in the body as compared to free drug (Mahapatro

and Singh 2011). Poly(D, L-lactide-co-glycolide) (PLGA) nanoparticles containing dexamethasone were formulated and conjugated with PDGF-BB (platelet-derived growth factor-BB [homodimer]) peptides. The nanoformulation was found to be biocompatible and stable, showed a sustained release over 14 days, and significantly increased cellular uptake (Kona et al. 2012).

1.3 Types of Nanomaterial Used in Drug Delivery

Nanomaterials are particles/molecules/structures of size ranging from 1 to 100 nm, although several marketed nanomedicines are in the range of 100–1000 nm. Nanomaterials should possess optimized physiochemical and biological properties as they are readily taken up by the cells in comparison to the bigger-size molecules and consequently aid in successful drug delivery system (Wilczewska et al. 2012). There are several types of nanomaterials such as polymeric nanoparticles (Chan et al. 2010), solid lipid nanoparticles (Mukherjee et al. 2009), liposomes (Akbarzadeh et al. 2013), and metal nanoparticles (Mody et al. 2010), which if investigated could be an interesting approach in drug delivery systems (Fig. 1.2). Some of the selected examples of drug-loaded nanomaterials used in the drug delivery system are shown in Table 1.3. Presently, more than 80 newly manufactured nanoformulations are investigated in preclinical and clinical trials (Huang et al. 2016).

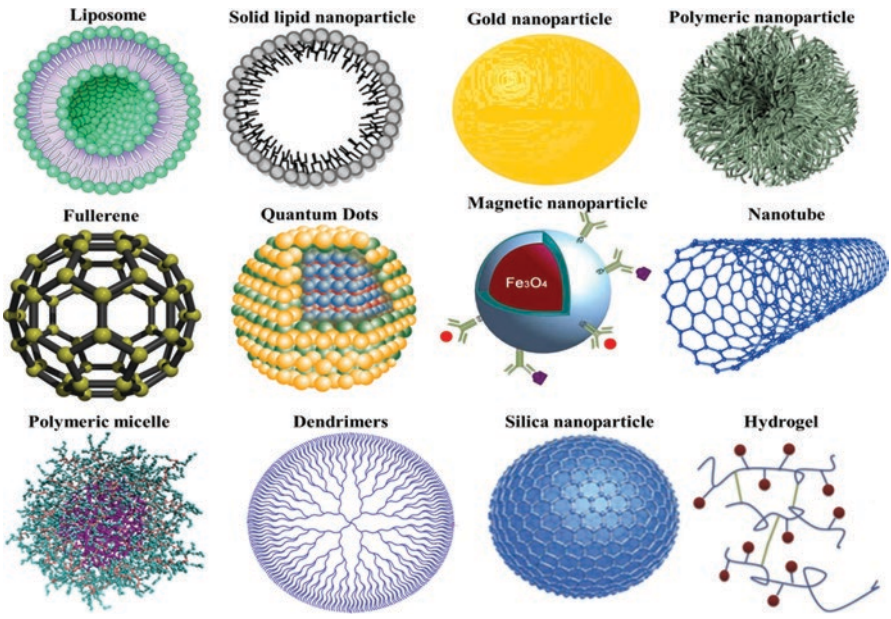

Fig. 1.2 Graphical representation of the different types of nanomaterial used in drug delivery

Table 1.3 List of selected nanoparticles used as drug delivery system

Sr. no.	Drug	Activity	Nanoparticle used	Synthesis method	Reference
1	9-Nitrocamptothecin	Anticancer	PLGA	Nanoprecipitation	Derakhshandeh et al. (2007)
2	Paclitaxel	Anticancer	PLGA	Interfacial deposition	Fonseca et al. (2002)
3	Cisplatin	Antitumor	PLGA-mPEG	Emulsion solvent evaporation	Moreno et al. (2010)
4	Dexamethasone	Anticancer	PLGA	Solvent evaporation	Gómez-Gaete et al. (2007)
5	Triptorelin	Anticancer	PLGA	Double emulsion solvent evaporation	Park et al. (2012)
6	Xanthones	Antileptospira	PLGA	Solvent displacement	Teixeira et al. (2005)
7	Haloperidol	Antipsychotic	PLGA	Emulsification-solvent evaporation	Budhian et al. (2005)
8	Ellagic acid	Antioxidant	PLGA	Emulsion diffusion-evaporation	Sonaje et al. (2007)
9	Savoxepine	Antipsychotic	Polylactic acid	Salting out	Kumari et al. (2010)
10	Progesterone	Steroid hormone	Polylactic acid	Solvent evaporation	Kumari et al. (2010)
11	Oridonin	Antitumor	Polylactic acid	Spontaneous emulsion solvent diffusion	Kumari et al. (2010)
12	Tamoxifen	Antiestrogenic	PEO-PCL	Solvent displacement	Kumari et al. (2010)
13	Sulfamethoxazole	Antibacterial	Chitosan	Solvent evaporation	Kumari et al. (2010)
14	Cyclosporin A	Antibiotic	Chitosan	Ionic gelation	Kumari et al. (2010)
15	Paclitaxel	Anticancer	Gelatin	Desolvation	Kumari et al. (2010)
16	Insulin	Antidiabetic	Gelatin	Ionic gelation	Kumari et al. (2010)
17	Taxol	Anticancer	Poly-caprolactone	Micelles	Kumari et al. (2010)
18	Clonazepam	Antiepileptic	Poly-caprolactone	Solvent evaporation	Kumari et al. (2010)
19	Docetaxel	Anticancer	Poly-caprolactone	Nanoprecipitation	Kumari et al. (2010)
20	Vinblastine	Anticancer	Poly-caprolactone	Emulsion	Kumari et al. (2010)

(continued)

Table 1.3 (continued)

21	Paclitaxel	Anticancer	PEG-b-PCL	Solvent evaporation	Banik et al. (2016)
22	Paclitaxel	Anticancer	PEG-PE	Lipid thin-film hydration	Banik et al. (2016)
23	Lamivudine	Anti-HIV drug	PLA/CS	Emulsion technique	Dev et al. (2010)
24	Tacrine	Anti-Alzheimer drug	Chitosan	Spontaneous emulsification	Wilson et al. (2010)
25	Carboplatin	Antineoplastic drug, ovarian, head, neck, and lung cancer	Sodium alginate	Ionic gelification	Nanjwade et al. (2010)
26	Doxorubicin	Antineoplastic agent	PEGylated PLGA	Surface modification technique	Park et al. (2012)
27	Capecitabine	Prodrug of fluorouracil, metastatic colorectal and breast cancer	CS-poly(ethylene oxide-g-acrylamide)	Emulsion cross-linking	Agnihotri and Aminabhavi (2006)
28	Diclofenac sodium	Anti-inflammatory	Xanthan gum+polyvinyl alcohol	Emulsion cross-linking	Ray et al. (2010)
29	Metoclopramide hydrochloride	Antiemetic	Gellan gum	–	Mahajan and Gattani (2010)
30	Nifedipine	Antihypertensive	Succinyl-chitosan /chitosan	Water-in-oil (w/o) emulsion cross-linking	Kajjari et al. (2013)

1.3.1 Polymeric Nanoparticles

Polymeric nanoparticles are structures of solid colloidal particles with a size dimension of 10 to 1000 nm (1 μm). These nanoparticles are prepared from synthetic polymers, such as polycaprolactone (PCL) (Dash and Konkimalla 2012), poly-D,L-lactide-co-glycolide (PLGA) (Sah and Sah 2015), and polylactic acid (PLA) (Mahapatro and Singh 2011) and/or natural polymers, like chitosan (Rampino et al. 2013), and alginate (Paques et al. 2014). Usually, these nanoparticles are capped with nonionic surfactants to reduce the interaction with the immunological system and interactions among the functional groups attached to the nanoparticles. They can be easily hydrolyzed and eliminated by producing biodegradable monomers like glycolic acid and lactic acid. Due to the higher surface area of nanoparticles, the drug molecule can easily be dissolved, encapsulated, or attached to the nanoparticle matrixes (Kumari et al. 2010). The application of biodegradable nanoparticles is among the best approaches in nanomedicine. The drug-loaded biodegradable

polymeric nanomaterials are safe in blood, nontoxic, and non-thrombogenic. They are non-immunogenic, are noninflammatory and do not stimulate neutrophils or influence the reticuloendothelial system. The nanometer size range increases efficacy after entering the cell membrane and provides stability in the bloodstream (Wilczewska et al. 2012). Polymeric nanoparticles are still in the preclinical research and also acquire the potential for targeted anticancer drug delivery. According to the methods of preparation, polymeric nanoparticles are classified as nanospheres and nanocapsules. The system in which the drug is confined to a cavity surrounded by a unique polymer membrane is known as nanospheres, whereas the matrix systems in which the drug molecule is physically and consistently dispersed are nanocapsules. The lowest toxicity was observed using PLGA for drug delivery. Such polymeric nanoparticles are compatible with the cells and tissues (Kumari et al. 2010). An example of drug delivery using polymeric nanoparticles is cisplatin, an anticancer molecule which has been loaded into copolymer PLGA-methoxy-PEG (PLGA-mPEG) nanoparticles. The in vitro results showed that cisplatin-loaded PLGA-mPEG nanoparticles passively targeted prostate cancer cells and showed less cytotoxicity compared to free cisplatin, but their passive targeting decreased toxicity. Through the use of fluorescence microscopy, the cellular uptake via internalization was confirmed. In addition to this, the in vivo mouse model also revealed that cisplatin blood levels were prolonged and sustained at therapeutic concentrations after intravenous administration (Malam et al. 2009). In addition to drug delivery, polymeric nanomaterials also play a role in gene delivery. The triblock copolymeric formulation of PEO_{20}-PPO_{69}-PEO_{20} poly(ethylene oxide)-poly(propylene oxide)-poly (ethylene oxide) with DNA nanoconjugates could be a potential carrier system. The plasmid DNA was found to be transported inside cells, which was further confirmed by the expression of genes within the cells (Daima et al. 2018).

1.3.2 Hydrogels

The hydrogels are three-dimensional macromolecule, which is an insoluble polymeric matrix comprised of hydrophilic components that are interconnected to absorb a large quantity of fluid. The higher water absorption capacity of hydrogels favorably simulates living tissues, in comparison to synthetic polymers that are soft textured and have low interfacial tension with an aqueous medium (Sharpe et al. 2014). In hydrogels, the ability to absorb fluid could be due to existing groups like –OH, -CONH, -COOH, and –SO3H, while their protection from dissolution arises from the cross-link between network chains. The hydrogels can be modified for a specific therapeutic action by forming a hydrogel complex. The network-forming hydrogels can protect the drug from the enzymes acting in the body and the low pH in the stomach. Few reports show that the mesh size of the hydrogels lies between 4 and 100 nm, which can be calculated experimentally or theoretically (Caló and Khutoryanskiy 2015). The unique characteristics of mechanical and degradation

properties of hydrogels make them useful in the drug delivery system. Hydrogels are mainly categorized into two classes:

(i) Permanent/chemical gel: When the gels form covalently cross-linked networks, they are known as "permanent" or "chemical gels."
(ii) Reversible/physical gel: If molecular association or interaction forces like hydrogen bonding, ionic bonding, or hydrophobic interactions are involved in assembling the network, they are known as "reversible" or "physical gels" (H. Gulrez et al. 2011).

Hydrogels can be formed using synthetic or natural polymers or a mixture of both. Synthetic polymers such as polylactic acid (PLA), polyethylene glycol (PEG), and polyvinyl alcohol (PVA) are precisely structured and have high mechanical properties, degradation, and release kinetics. Natural polymers like alginate, chitosan, agarose, collagen, and cellulose may also have efficient mechanical properties and could invoke immunogenic or inflammatory response. Additionally, they also have compatible physiochemical properties as well as are nontoxic, which is due to their natural source. Recent reports showed significant research on the utilization of hydrogels in the areas of cardiovascular implants, wound dressing, and controlled release of the drug. The antibacterial activity of curcumin-loaded nanoparticles has been observed by encapsulating in protein hydrogels (Vimala et al. 2014).

1.3.3 Metal Nanoparticles

Metal nanoparticles show many applications in diverse fields of biomedical science including diagnostic assay development (Baptista et al. 2008; Selvan et al. 2010), as probes for electron microscopy to see intracellular organelles/compartments, detection and therapies, thermal ablation (Hirsch et al. 2003), and radiotherapy enhancement (Hainfeld et al. 2004), along with drug and gene delivery (Han et al. 2007). Metal nanoparticles are composed of different shapes and sizes ranging from 10 to 100 nm, which are explored in diagnostic and drug delivery systems. The common metal nanoparticles used in biomedical sciences so far are gold, silver, nickel, zinc oxide, iron oxide, and magnetic nanoparticles (Díaz and Vivas-Mejia 2013). The use of metal nanoparticles especially gold nanoparticles attained a lot of interest in the field of biomedical science for the delivery of therapeutic agents to their targets (Kong et al. 2017). These nanoparticles utilize their unique physiochemical properties for delivering and discharging therapeutic agents. The gold core is biocompatible, non-cytotoxic, truly inert, stable, and nontoxic in nature. Another uniqueness is their method of synthesis; monodisperse nanoparticles can be obtained with core sizes which range from 1 to 150 nm (Ghosh et al. 2008; Alaqad and Saleh 2016). The presence of a negative charge on the surface of the nanoparticle is easily modified and functionalized using biological components like DNA, peptides, and antibiotics through either covalent or non-covalent bonding. The gold nanoparticle becomes the potential nanocarrier for various applications in

the field of biomedical sciences including biosensing, drug delivery, molecular imaging, etc. The spherical-shaped gold nanoparticles are the most widely used gold nanostructures in drug delivery (Ghosh et al. 2008; Kong et al. 2017). Generally, gold nanoparticles are efficiently synthesized with single high dispersion by reducing gold salts with sodium citrate, where sodium citrate acts as a stabilizing agent (Kong et al. 2017). These nanoparticles may also be synthesized using plant extracts as reducing agents. The common plants used as reducing agents are *Morinda citrifolia*, *Pelargonium graveolens*, *Punica granatum*, *Salvia officinalis*, *Lippia citriodora*, etc. (Elia et al. 2014). Gold nanoparticles have shown potential to deliver various recombinant proteins, drug molecules, and vaccines, which play a crucial role in the therapy of endocellular diseases. The use of PEGylated gold nanoparticles in human cancer cells and in xenograft tumor mouse models for targeting tumor can be detected spectroscopically by surface-enhanced Raman scattering (SERS, Qian et al. 2008). It was reported that the doxorubicin attached to 30 nm gold nanoparticle allows intracellular release of doxorubicin from the nanoparticle when it enters into acidic organelles, thereby increasing the intracellular doxorubicin concentration and enhancing therapeutic action into their target with controlled release via biological stimuli or light activation (Wang et al. 2011).

Silver nanoparticles have also been widely used in drug delivery, nanomedicine, cosmetics, air, and water filtration and electronic application due to its potential biological, chemical, and physical characteristics and huge commercialization. They are also applied in daily-use commercial products due to its broad spectrum of antimicrobial activity. Additionally, due to the properties of individual plasmon optical spectra, it is useful in the biosensing application (Alaqad and Saleh 2016). Mostly, metal particles are synthesized by two approaches, i.e., top-down and bottom-up, which involve physical, chemical, and biological methods. The most common approach used for the synthesis of silver nanoparticles is the chemical reduction method. The reduction of silver nanoparticles was performed using sodium citrate, ascorbate (Firdhouse and Lalitha 2015), sodium borohydride (NaBH4) (Prabhu and Poulose 2012), elemental hydrogen, polyol process, ascorbic acid, poly(ethylene glycol)-block copolymers (Nasrollahzadeh 2014), N,N-dimethylformamide (DMF) (Beyene et al. 2017), hydrazine, and ammonium formate (Hussain et al. 2011) in the aqueous or nonaqueous solution. The other chemical approach is the microemulsion method which change the properties of particles like, particle size control, homogeneity, morphology, geometry, and surface area. Silver nanoparticles are also prepared using the green synthesis method, i.e., by using biological components like bacteria, fungi (yeast), and plant extracts. The application of microorganisms has achieved a lot of attention due to the production of silver nanoparticles (Beyene et al. 2017). Among the physical methods, evaporation-condensation and laser ablation are two of the most important approaches. Usually, silver nanoparticles are synthesized in the range of 1–100 nm. The nanosize of nanoparticles provides a large surface area and enhances its effect which ultimately increases the penetration potential through cells and tissues. Silver

nanoparticles have the potential to enter the circulatory system and cross the blood-brain barrier (Prabhu and Poulose 2012). Among the metal nanoparticles, magnetic nanoparticles are also particularly used in the area of cancer treatment. Magnetic nanoparticles have extensive properties like easy preparation, facile functionalization, biocompatibility, and responsiveness to physiological conditions, making them favorable for targeted drug delivery using the external magnetic field. The materials used for the preparation of the magnetic part are oxides of iron, cobalt, and nickel and are generally combined with various metals like copper, strontium, zinc, iron, nickel, and barium (Kudr et al. 2017). According to the literature, the most commonly used material for the preparation of magnetic particles is superparamagnetic iron oxide nanoparticles (SPION) or iron oxide nanoparticles (ION), due to their attractive biocompatibility. Some iron oxide nanoparticles like Gastromark, Resovist, Feridex, and Sinerem have been approved by the USFDA/European Commission (EC) as magnetic resonance imaging agents, and ferumoxytol is used as an iron supplement for treating iron deficiency. Based on the size, crystallinity, and easy formulation, several methods evolved and are selected for the production of the core region of magnetic nanoparticles. These methods include thermal decomposition, hydrothermal deposition, coprecipitation, microemulsion, direct reduction, and polyol synthesis (Huang et al. 2016). To eliminate drug solubility problem for systemic delivery, magnetic nanoparticles can be easily functionalized using various organic, inorganic, and carbon nanotubes (Fadel et al. 2014). The magnetic nanocarrier was developed in which a complex of doxorubicin (Dox) attached to the Fe_3O_4 nanoparticle was encapsulated in polyethylene glycol functionalized porous silica nanomaterial. As a result, the drug-loaded magnetic composite system presented a slow release of the drug compared to the Dox-Fe_3O_4 nanomaterial system. The polyethylene glycol polymer allowed the composite complex (Dox-Fe_2O_3) to be eliminated from the reticuloendothelial system, allowing the drug to be carried out over prolonged duration (Chen et al. 2010).

Titanium oxide nanoparticles are broadly manufactured nanomaterials in the world. Their addition to composites led to enhancement in their mechanical properties and reduced the risk of bacterial infections. These nanomaterials are frequently used in endoprostheses and scaffolds for reconstruction of bone tissue (Tautzenberger et al. 2012). Titanium oxide nanoparticles have been used in industrial and consumer products due to their high catalytic activities. The increment in the catalytic activity may be due to their small size, which enhanced surface area per unit mass. However, similar properties of titanium oxide nanoparticles may have different biological activities, which may become a challenge for humans (Tsuji et al. 2006). It was reported that after oral administration, titanium oxide nanoparticles induce DNA double-strand breaks in bone marrow cells (Chen et al. 2014). Titanium oxide nanoparticles were found to be toxic to organs and badly affected the knee joints in rabbits (Wang et al. 2009). According to some reports, titanium oxide nanoparticles were considered to be safe initially, but it may be harmful to human health.

1.3.4 Silica Nanoparticles

The mesoporous silica nanomaterials are known to be a promising and novel nanomaterial that has been broadly used as a delivery reagent due to the different chemical properties, thermal stability, large surface area, pore volume, controllable size, and biocompatibility. The mesoporous structure with a pore size of 2–50 nm promotes efficient loading of the drug and their controlled release to the target site (Bharti et al. 2015; Wang et al. 2015). The broad surface area of silica nanoparticles allows it to bind to the different types of functional moieties for targeting the therapeutic drug to its site of action. These silica nanoparticles are used in many areas like target drug delivery, diagnosis, cellular uptake, and biosensing. Silica is also authorized as "generally recognized as safe" (GRAS) by the USFDA and broadly used for cosmetics and food-additive purposes (Watermann and Brieger 2017). Due to the porous nature of silica nanoparticles, they are able to encapsulate a drug molecule with two advantages. First, it protects the drug from early degradation and identification by the immune system and makes the lower concentration of dose more effective (Gao et al. 2011). Second, it helps in minimizing side effects (De Jong and Borm 2008). Silica nanoparticles can be prepared using synthetic or natural sources. The synthetic, low-cost sources include sodium silicate solution (SSS) and tetraethyl orthosilicate (TEOS). These particles are prepared by the application of hydrochloric acid (HCl) as a precipitating agent, along with carbon dioxide (Zulfiqar et al. 2016). Some natural waste materials like rice husk, bamboo leaves, groundnut shell, and sugarcane bagasse are also used for the synthesis of sodium silicate solution (SSS) (Vaibhav et al. 2015). In the recent years, the formulation of ordered mesoporous silica materials has been reported including sol-gel processes to create common SBA-15 (Santa Barbara Amorphous) and MCM-41 (mobile crystalline material, Argyo, et al. 2013). In the early 1990s, the M41S family was the first to be reported among ordered mesoporous silica (Kresge et al. 1992). A considerable number of in vitro experiments have been conducted to gain insights regarding the viability of mesoporous silica nanomaterials as drug carriers. The diverse forms of the drug – like propidium iodide, colchicine, chromobodies, phalloidin, calcein, or a rhodamine derivative – get adsorbed into mesoporous silica nanomaterials, which were subsequently sealed by a supported lipid bilayer and show effective loading capacity. Tian and coworkers proved the efficacy of various drug molecules enhanced by encapsulation in MSN carriers through in vitro experiments (Argyo et al. 2013).

1.3.5 Micelles

In the recent past, polymeric micelles attained importance in the area of drug delivery. They are also used as a carrier for poor solubility drug, genes (Shen et al. 2009), and imaging agents (Kedar et al. 2010). The formulation of micelles is also consid-

ered as an effective method for delivering hydrophobic drug (Antoine and Jonathan Lawrence 2013). Usually, polymeric micelles are self-assembled, are 5–100 nm in size, and made up of the inner core and the outer shell. These are formed from the association of blocked copolymers with the capacity to increase the solubility rate of the hydrophobic molecule. Each part of the polymeric micelle plays an important role, i.e., the inner core encapsulates the hydrophobic drug and serves as a reservoir of the drug molecule. The outer shell is responsible for protecting the drug from the biological component in the blood. The outer shell, also known as corona, preserves the polymeric micelles against acceptance in vivo by the reticuloendothelial system (RES), resulting in prolonged blood circulation (Movassaghian et al. 2015). The polymeric micelles can be synthesized using various types of copolymers by forming di- and triblock copolymers. The poly(lactide-co-glycolide) and polyethylene glycol are some of the frequently used materials in the production of polymeric micelles due to their nontoxic nature and FDA certification. There are various methods used for the preparation of polymeric micelles like emulsification, solvent evaporation or nanoprecipitation, and salting out (Antoine and Jonathan Lawrence 2013). Due to low incorporation of the drug and drug loading capacity, their targeting ability is limited. Important characteristics of micelles are the extent of controlled drug release by external factors like temperature, pH, and ultra-sonication of enzymes (Díaz and Vivas-Mejia 2013). According to a few recent reports, USFDA-approved compounds are relatively few like Genexol-PM, Estrasorb, and Flucide (Bobo et al. 2016). Polymeric micellar carriers for the encapsulation and delivery of amphotericin B were developed to avoid drug distribution at the site of drug toxicity (Lavasanifar et al. 2002).

1.3.6 Liposomes

Liposomes are spherical vesicles, with sizes ranging from 30 nm to several micrometers. These are made up of one or two layers of vesicles surrounding aqueous units which consist of natural or synthetic phospholipids (Fakhravar et al. 2016). Liposomes were the first most considered nanomaterial practiced in the area of medicine since Bangham described them in 1961 (Díaz and Vivas-Mejia 2013). In addition to medicine, liposomes have been used in many areas like biology, food, biochemistry, and cosmetics (Fakhravar et al. 2016). These are commonly used delivery systems for different functional moieties like small molecules, small and long nucleic acids, peptides, and proteins. Liposomes can be prepared using various phospholipids like phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, and other molecules such as cholesterol (Abreu et al. 2011), which helps in the effective entrapment of the drug. As a drug nanocarrier, it increases the drug residence time in the bloodstream, by supporting slow release and drug stability under in vivo conditions. The synthesis of the liposome mixture is complex due to complication in attaining particle size and effective encapsulation efficiency. Several methods were developed, but still the problem of stabilization,

particle size control, less drug encapsulation, and reproducibility persists. To make the preparation method effective, a combination of methods has been adopted (Yang et al. 2013) like ultrasonic and mechanical methods such as film method, methods involving fusion of prepared vesicles or transformation of size by freeze-thaw extrusion, replacement of organic solvent, and the dehydration-rehydration method.

Along with this, the dual asymmetric centrifugation, supercritical fluid technology, cross-flow filtration technology, membrane contactor technology, and freeze-drying technology have also been selected for the preparation of liposome (Huang et al. 2014). The addition of specific functional groups such as polyethylene glycol improves the specificity of the nanoparticle. For example, when liposomes are covalently bound to PEG for reducing the identification by macrophages, the stability and circulation half-lives were increased. Several liposomal formulations have been approved by the USFDA like Doxil, DaunoXome, and Abraxane (Bobo et al. 2016) and many are in different clinical trial phases like nano-liposomal CPT-11, SPI-077, and CPX-351 (Díaz and Vivas-Mejia 2013). Doxil and Myocet are the first USFDA-approved liposomal formulations for the treatment of cancer. Both formulations showed an increased half-life in circulating blood than the free Myocet. However, among the two, Doxil showed much higher circulation time in blood compared to Myocet. It was reported that the hydrophilic prodrug-loaded PEG-coated liposomes were prepared to eradicate the instability. It was also observed that the chemical bonds in the prodrug of paclitaxel and the liposomes were reliable in in vitro hydrolysis using rat plasma and there was no problem of crystallization (Lim et al. 2013a).

1.3.7 Solid Lipid Nanoparticles

The solid lipid nanoparticles (SLNs) with a spherical shape and an average diameter between 10 and 1000 nm have been employed as a novel approach for drug delivery systems. The SLN provides different properties like small size and can deliver a lipophilic drug with high drug loading, low toxicity, and a large surface area (Lim et al. 2013a). The lipids which are generally used in the preparation of SLNs include fatty acids (stearic acid), steroids (cholesterol), waxes (cetylpalmitate), monoglycerides, diglycerides, and triglycerides (tristearin). In addition to these lipids, different polymers and surfactants are also used for providing stability and avoiding aggregation. The selection criteria of the lipids and surfactants influence the physiochemical parameters of the drug-loaded SLNs. Few commonly used methods for the preparation of solid lipid nanoparticles are hot, high-pressure homogenization technique and cold, high-pressure homogenization technique, solvent emulsification/evaporation, high shear homogenization and ultrasound, and microemulsion. For example, many hydrophobic and hydrophilic drugs like doxorubicin (Subedi et al. 2009), paclitaxel (Baek et al. 2016), tobramycin (Cavalli et al. 2002), and cyclosporine A (Sawant et al. 2008) have been encapsulated in the SLN system. Subedi et al. developed doxorubicin-loaded solid lipid nanoparticles (SLN-DOX)

using biocompatible compounds and tested their in vivo therapeutic effects (Subedi et al. 2009). Compared with that of free DOX, SLN-DOX showed the potential to serve as a useful therapeutic approach to overcome the issues of chemoresistance of adriamycin-resistant breast cancer (Lim et al. 2013a). In another study, naringin-loaded SLNs showed significant improvement in the relative bioavailability after administration via pulmonary instillation (Wu et al. 2016).

1.3.8 Fullerenes and Carbon Nanotubes

The fullerenes and carbon nanotubes (CNTs) belong to the family of carbon nanomaterials or carbon allotropes. Carbon nanotrophs have captured the attention of researchers and opened many opportunities in the area of nanotechnology due to their unique physiochemical properties and size-dependent functions (Tripathi et al. 2015). Carbon nanomaterials are nanomolecular carbon cages with various advantages like functional modifications, stability, high drug carrying capacity and utility of mixing hydrophobic and hydrophilic molecules. These properties are essential for the improvement of potential drug molecules in systemic drug delivery (Iohara et al. 2011; Yamashita et al. 2012). Fullerenes were discovered by Harold W. Kroto, Robert F. Curl, and Richard E. Smalley, who also received the Noble Prize in the area of chemistry in 1996. Fullerene is a closed cage-like structure made up of 20 hexagonal and 12 pentagonal rings, in which each carbon atom was sp^2 hybridized and attached to three carbons. Fullerenes and CNTs are mostly identical in their structure, i.e., systematically consist of a large π-attached carbon chain. Fullerenes which are spherical are commonly known as buckyballs, whereas the cylindrical-shaped fullerenes, which are wrapped by a graphene sheet, are called as buckytubes or CNTs. CNTs are large cylindrical molecules composed of hexagonal rings of sp^2-hybridized carbon atoms. These are characteristically microscopic rather than nanoscopic, usually greater than 100 nm (Tripathi et al. 2015). In addition to CNTs, fullerenes have also shown wide application in drug delivery mostly because of its small size (~1 nm) and biological activity. They can easily enter tissues and organelles. They have diverse functional groups which allow accurate grafting to enhance their activity. The functionalization of fullerene with hydrophilic molecules increases its aqueous solubility, making it competent to deliver drug or gene to cellular system (Bolskar 2016). The drug named doxorubicin (DOX) was attached to fullerenes and carbon nanotubes (Meng et al. 2012) with the aim of mitigating DOX-induced toxic side effects and enhancing drug delivery (Blazkova et al. 2014). The SWCNT-paclitaxel (PTX) composite system was prepared by adjoining PTX to functionalized polyethylene glycol SWCNTs through ester bonds. The higher tumor uptake of PTX and increase ratios of a tumor to normal organ PTX uptake for SWCNT-PEG-PTX in comparison with Taxol and PEG-PTX, along with the higher efficacy of tumor suppression and low side effects, were observed (Liu et al. 2008).

1.3.9 Dendrimers

Dendrimers are treelike structures, synthetic in nature, three-dimensional, radially symmetric molecules with well-defined, homogeneous, and monodisperse structure of nanometer dimensions (Abbasi et al. 2014). They are made up of three different components, a central core which may be a single atom or a group of atoms, a building block which consists of repeating units and an exterior part which is composed of multiple functional groups (Nanjwade et al. 2009). Different types of dendrimers have attracted attention in drug delivery such as poly(amidoamine) (PAMAM), poly(propyleneimine) (PPI), and poly(L-lysine) (PLL). These are mainly synthesized using divergent and convergent approaches. The divergent method was developed by Tomalia in 1996. It involves the growth of dendrimers emerging from the focal core and gathering of monomers radially, one branch overlaping the new branch, depending upon certain dendritic rules. The convergent method was created by Hawker and Fréchet following a "convergent growth process". In this method, different dendrons are reacted with the multifunctional core to achieve a product (Nanjwade et al. 2009). The optimized three-dimensional structure of the dendrimers provides different properties such as functional groups at the periphery, globular shape, nanometer size range, narrow polydispersity, and the hydrophobic or hydrophilic gap in the interior (Noriega-Luna et al. 2014). In case of drug delivery, drug molecules are either covalently attached to the groups on the periphery or non-covalently entrapped in the interior cavity of the dendrimers. Many reports show that anticancer drugs like camptothecin, 6-mercaptopurine, methotrexate, adriamycin, 5-fluorouracil, and paclitaxel are entrapped into the PEGylated polyamidoamine (PAMAM) dendrimer. It demonstrates a significant improvement in water solubility, storage stability, minimization of side effects, and antitumor activity. It was demonstrated that 5-fluorouracil- and cisplatin-loaded PEGylated PAMAM dendrimers showed high efficiency for loading and releasing against cancer cell lines (Tran et al. 2013). In another example, Barker and coworkers formulated dendrimers attached with fluorescein and folic acid for imaging and therapeutic purposes. In this study, dendrimers were combined with complementary DNA oligonucleotides to produce clustered molecules that target cancer cells overexpressing high-affinity folate receptors (Díaz and Vivas-Mejia 2013).

1.3.10 Quantum Dots

Quantum dots are fluorescent semiconductor nanoparticles with a size range of 2–10 nm, exhibiting photostability and spectral properties. They mainly consist of elements such as Cd, Pb, and Hg. The unique and fascinating optical properties of the quantum dots, like high yield and good chemical and photostability, make them useful materials as luminescent nano-probes and vehicles in biological applications (Matea et al. 2017). The quantum dots contain a core and a shell, which cover the

core region from oxidation and increase their yield. These are extensively used for labeling, detection, and bio-imaging. It shows many advantages over traditional fluorescent dyes and proteins like being brighter and have photostability, broad luminescence excitation spectra and definite symmetrical emission spectra with large Stokes shift (Bilan et al. 2016). Quantum dots, which transmit light in the near-infrared region, are suitable for imaging in thick tissues in in vitro and in vivo conditions (Li et al. 2014; Rizvi et al. 2014). In medical science, the photophysical properties of functionalized water-soluble quantum dots are beneficial when used with biomolecules like proteins, drugs, or antibodies (Bilan et al. 2015). However, it faces many challenges such as tough surface chemistry and moderately huge sizes. The application of quantum dot in clinical research face severe issues of toxicity (Sun et al. 2013), even though there are many examples of drug-loaded quantum dots used in drug delivery. The doxorubicin was loaded onto pH-responsive ZnO-QDs. It was functionalized with poly(ethylene glycol) and hyaluronic acid for targeting the overexpressing glycoprotein CD44 in cancer cells (Cai et al. 2016). Yang et al. synthesized quercetin-loaded CdSe with ZnS quantum dots as anticancer and antibacterial nanostructures. It exhibited more efficacy against drug-resistant *Escherichia coli* and *Bacillus subtilis* compared to quercetin and CdSe nanoparticles. The anticancer activity assay showed enhancement of two- to sixfold of cytotoxicity than the quercetin and CdSe quantum dots (Yang et al. 2017).

1.3.11 Protein Nanocarriers

Protein nanocarriers are considered to be GRAS (generally regarded as safe) drug delivery systems due to their extraordinary properties like high nutritional value, biodegradability, plentiful renewable sources, non-antigenicity, and exceptional binding capability of several drugs. Protein generally has fewer chances of opsonization by the reticuloendothelial system (RES) via steric barrier and large functional properties like gelation, foaming, and water binding capacity (Elzoghby et al. 2011; Elzoghby et al. 2012). Due to the presence of the multiple functional groups in polypeptide sequences, the protein nanoparticles can be modified to produce different interactions with therapeutics, offering chances for reversible binding of therapeutic molecules and specific targeting to the site of localization (Elzoghby et al. 2012). Protein nanoparticles can be easily developed, scaled up, and hydrolyzed by digestive enzymes, which causes bioactive peptides that led to physiological effects in vivo. Gelatin is a denatured protein, which is considered to be GRAS by the FDA, in pharmaceuticals, food products, and cosmetics. The successful delivery of several drugs, like anticancer (Yeh et al. 2005), antimalarial (Bajpai and Choubey 2006), anti-HIV (Jain et al. 2008), anti-inflammatory (Kumar et al. 2011), and antimicrobial (Nahar et al. 2008), using gelatin nanoparticles has already been reported.

Similarly, albumin nanoparticles have shown to be nontoxic in nature, biodegradable and biocompatible, and easily metabolized by enzymes. It plays a critical

role in the development of a pharmacokinetic profile along with the targeting efficacy of several drugs as it has a half-life of 19 days in the blood circulation (Elsadek and Kratz 2012). The drug named Abraxane® (paclitaxel-albumin nanoparticles) was used for treating metastatic breast cancer (Elsadek and Kratz 2012).

1.4 Nanomaterials in Drug Delivery: Some Success Stories

1.4.1 Nanoparticles for Dermal and Transdermal Drug Delivery

The delivery of therapeutic agents through the skin is an exciting area of research, due to its property of acceptance and ease of access. Many of the therapeutic agents are not delivered systemically through various modes of administration, due to various drawbacks of size with delivery beyond the epithelium and early degradation (Fig. 1.3, Schoellhammer et al. 2014). As the primary function of the skin is to provide a protective barrier between the body and external environment, many of the therapeutic agents like macromolecules and drugs are administered by hypodermic needles (Walter et al. 2010). But these injections have certain drawbacks like pain and phobias of the needle, transfer of infectious disease by reusing the needle,

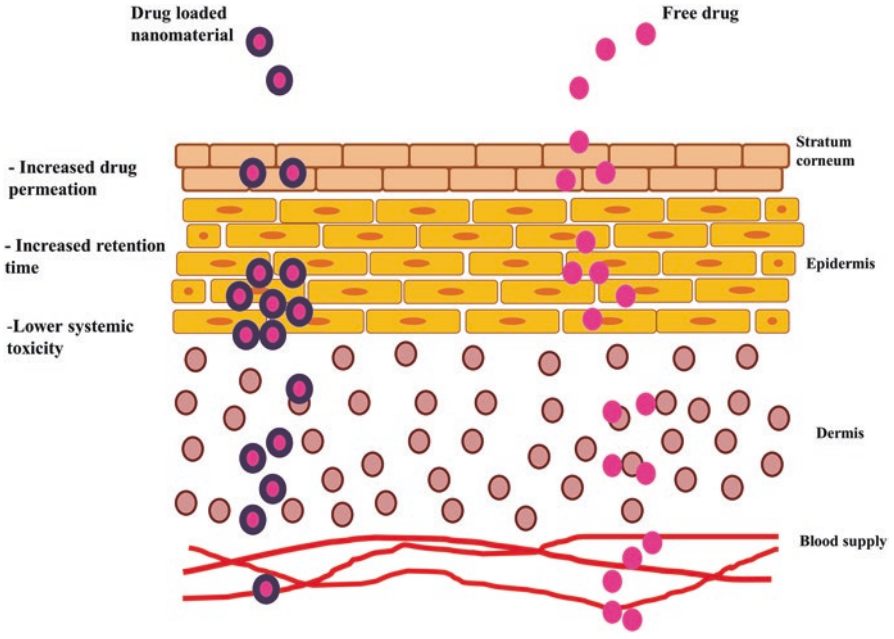

Fig. 1.3 Schematic representation of the comparison between the free drug molecule and the therapeutic loaded nanocarrier

and sometimes, an unexpected injury. Another problem is burning; it is usually a skin wound occurring due to exposure to heat like fire, hot liquid, and gases. According to the World Health Organization, about 265,000 people died due to thermal burns in low- to middle-income countries every year. The prevention and treatment of infection due to thermal burns (Mofazzal Jahromi et al. 2018) could be achieved by the delivery of the drug through the dermal/transdermal system route. The dermal/transdermal drug delivery system (TDD) is one of the popular approaches which enables the successful implementation of therapeutic agents. The transdermal route offers several advantages of bypassing the first-pass metabolism, enhancing the retention time, boosting patient acceptance, and increasing the efficacy of the drug (Alkilani et al. 2015). The drug concentrations can be adjusted to control the release of the drug for a prolonged time and reduce repetitive dosing, in which the TDD system is a useful area in the field of research. Scopolamine patch, used for motion sickness, was the first USFDA-approved transdermal drug in 1979 (Palmer et al. 2016). *The cost of transdermal drug delivery in the market was 12.7 billion dollars in 2005 and was expected to reach $32 billion in 2015.* There are many success stories behind the use of nanomaterials in dermal/transdermal drug delivery mentioned below.

At the beginning of the 1990s, many of the unique carrier systems have considerable attention which can be used topically like silver nanoparticles, cellulose, and chitosan (Ghanbarzadeh et al. 2015). The skin is the largest organ in the human body and composed of well-defined layers, i.e., the epidermis and dermis. In case of skin diseases, the main focus is on the entry of the drug into viable skin layers without disturbing systemic circulation to avoid off-target effects (Pariser 2009). Several nanosized nanomaterials like titanium dioxide, zinc oxide, silica nanoparticles, and fullerenes have already been added to cosmetic formulations to protect the skin against harmful ultraviolet rays or act as desiccants or free radical scavengers (Xiao et al. 2006; Contado 2015). Nanomaterials are found to enter the skin through three pathways: intercellular, intracellularly through corneocytes, or through hair follicles, through pores of the sweat glands (Fig. 1.4, Baroli et al. 2007). Several polymeric nanoparticles can be prepared by coating with various materials through innovative methods. Few reports show that keratinocyte growth factor (KGF) is a strong growth factor responsible for reepithelization of skin wounds. It was reported that KGF present in self-assembled nanoparticles is activated for quick wound healing, which ultimately enhanced reepithelization and regeneration of the skin (Feng et al. 2014). The epidermal growth factor (EGF)-loaded PLGA was prepared and used for the treatment of deep wounds and gave the maximum level of fibroblast proliferation (Yüksel et al. 2016). Many of the drug molecules suffer from few limitations like instability, insufficient penetration through the skin, and severe side effects. The main purpose of the topical and dermatological dosage is to deliver the drug molecule to the stratum corneum. The solid lipid nanoparticles increased the encapsulation efficiency and prolonged stability of hydroquinone, resulting in skin localization and minimum penetration in the receptor (Ghanbarzadeh et al. 2015).

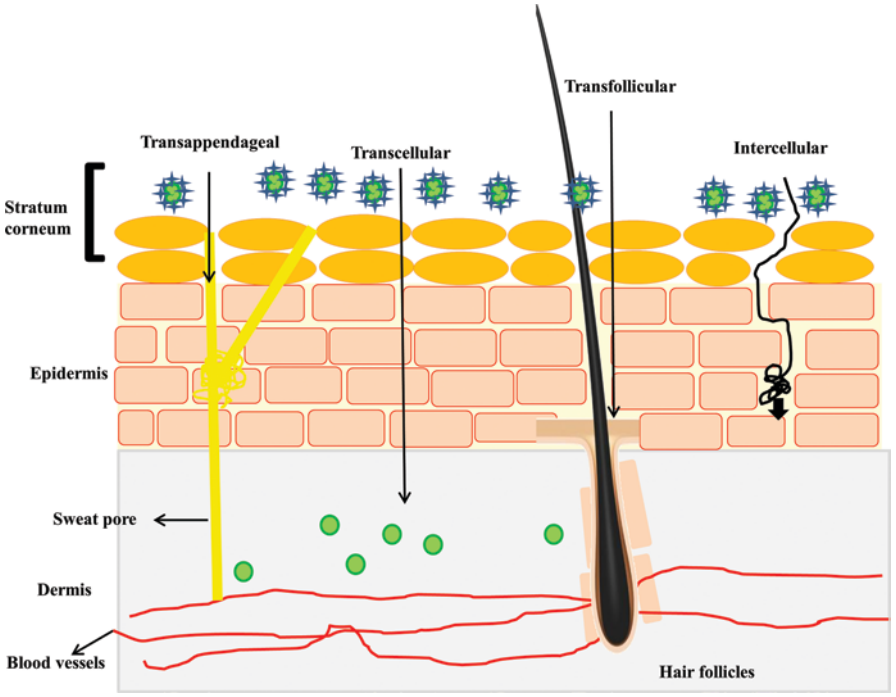

Fig. 1.4 Schematic representation of several routes of permeation through the skin during therapeutic delivery

Chitosan nanoparticles are usually applied in the form of dressings and bandages for an increment of wound healing rate (Baxter et al. 2013).

Several studies concluded that chitosan nanoparticles could promote wound healing by enhancing the activity of inflammatory cells and stimulating fibroblasts and osteoblasts. The loading of curcumin into the nanomaterial is a beneficial delivery route as curcumin faces the problem of aqueous solubility. In one study, curcumin-loaded nanomaterials were observed to have high antibacterial activity to *P. aeruginosa* and *S. aureus*, which help in the treatment of the skin infections in mouse wounds (Mofazzal Jahromi et al. 2014). The alginate-silver composite and antibiotic-loaded alginate oligomer have been employed as antimicrobial components against some microorganisms like *Enterococcus faecalis*, *P. aeruginosa*, and *E. coli* (Thomas et al. 2011). The hydrogels in combination with other nanoparticles like chitosan, keratin, polyvinyl alcohol, polyethylene glycol, fibrin, dextran, and antibiotics can lead to the transformation of the skin in case of burns (Anjum et al. 2016). The cumulative drug release capacity of the tetracycline hydrochloride-loaded hydrogels increased by 80% over 48 h, with enhanced antimicrobial effect against Gram-negative and Gram-positive bacteria, along with the reduction of scarring when applied over wounds (Ahmed 2015). The eucalyptus oil-entrapped chitosan film in combination with nanoemulsion showed a good antibacterial effect

against *S. aureus*. The dihydroquercetin-encapsulated liposomes could enhance antioxidant activity, along with a reduction in the necrotic area, in the burned region of the skin, which ultimately improved healing (Naumov et al. 2010). The nanoencapsulation of apigenin showed better effects compared to the free apigenin case of cancer. The apigenin was previously applied in mice to reduce the number and size of the tumors in the skin induced by chemical carcinogens or by UV exposure in vivo. Some drugs like doxorubicin, cisplatin, and oxaliplatin encapsulated in liposomes have shown a rise in the drug's cytotoxicity and reduction in their side effect, which is due to direct targeting. It was also observed that delivering doxorubicin (chemotherapeutic agent)-encapsulated gold nanoparticle is efficient against melanoma cell line. The etoposide-loaded cholesterol-rich nanoemulsion in a mouse model of melanoma minimizes side effects and improves inhibition activity of tumor growth and increment in the tolerance dose (Prete et al. 2006). Tumor reduction up to 60% was observed using doxorubicin nanomaterial along with an antibody against CD44 by targeting malignant cells. The magnetic nanostructure-loaded albumin/drug has remarkable therapeutic effects in the treatment of skin cancer, along with increment in the efficiency to hinder tumor growth (Misak et al. 2013).

1.4.2 Nanoparticles for Ocular Drug Delivery

The ocular drug delivery system is the biggest challenge in the area of pharmaceutical science. The uniqueness of the eye structure and its protective barrier inhibit the administration of the drug at their target site. The structure of the eye is composed of the anterior and posterior segments. The anterior segment comprises of the approximately one-third part, whereas the other part is filled up by the posterior segment. The anterior segment includes tissues like cornea, aqueous humor, iris, conjunctiva, ciliary body, and lens. The back portion of the eye known as the posterior segment includes the choroid, sclera, neural retina, retina pigment epithelium, optic nerve, and vitreous humor. Drug deliveries to the eye are generally through the anterior and posterior segments. Some possible nanocarriers and route of cellular uptake during ocular drug delivery are shown in Fig. 1.5. Most of the drug is washed out rapidly from the eye through tear dilution, lacrimation, and tear turnover, diminishing its bioavailability (Gaudana et al. 2009). Most of the times, applications of eye drops cause detrimental side effects including inflammation, blurred vision, and cellular damage which led to discomfort. Also, the human cornea consists of the epithelium; the substantia propria and endothelium inhibit ocular drug delivery. Due to several factors, only 5% of the drugs are administered through the eye (Le Bourlais et al. 1998; Gaudana et al. 2009). To increase the bioavailability and to prolong the retention time, emulsions, liposomes, micelles, polymeric inserts, and nanoparticle suspensions have been explored in ocular drug delivery (Yuan et al. 2015). Presently, nanomicellar formulation-based ocular drug delivery is gaining increased interest, due to its facile method of preparation, small size, and high drug encapsulation capacity. The nanomicellar formulations are also helpful in increas-

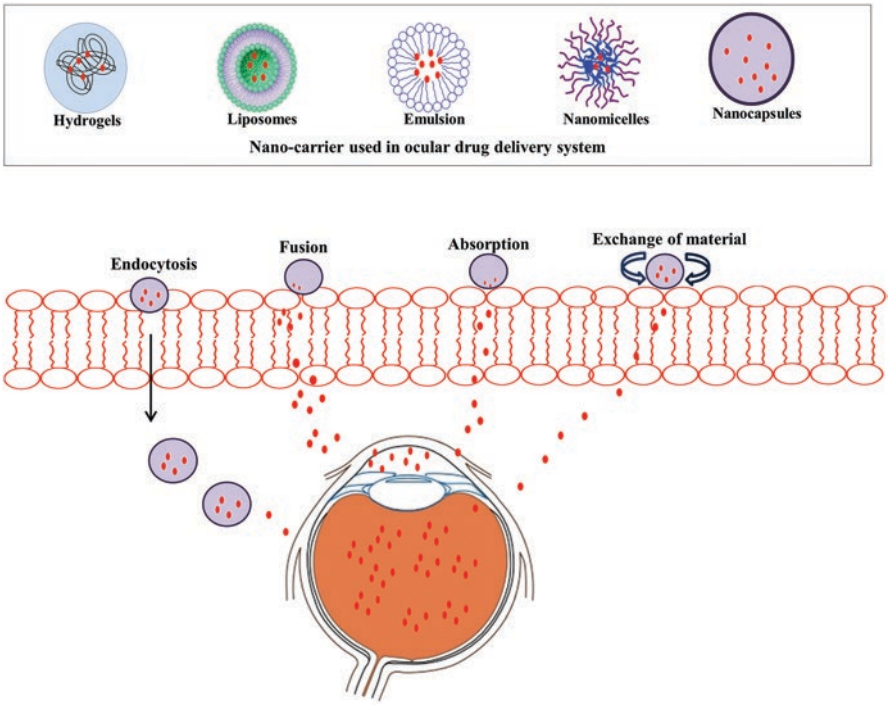

Fig. 1.5 Schematic representation of the nanocarriers used in ocular drug delivery and their possible routes of drug delivery

ing the bioavailability of therapeutic agents in ocular tissues, resulting in better conclusion. Until now, various proof studies have been regulated to explore the relevance of ocular drug delivery (Civiale et al. 2009; Cholkar et al. 2012). Several researchers employed nanomicelles for ocular gene delivery. The copolymeric micelle, poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), was employed for the successful DNA transfer. The ocular bioavailability of diclofenac sodium was increased using solid lipid nanoparticle which is composed of a homolipid from *Capra hircus* (goat fat) arranged with phospholipon 90G® (Seyfoddin et al. 2010). Several formulations were developed to increase ocular bioavailability. Commercial products like Timoptic-XE® and Zirgan®, based on in situ gelling, have been put in motion in the United States for the treatment of ocular diseases. Timoptic-XE® is an ion-activated gel that entraps timolol for the treatment of glaucoma.

Zirgan® is a pH-sensitive hydrogel which is encapsulated with ganciclovir for herpes simplex virus in the eye (Shi et al. 2015). Axitinib-loaded nanowafer under preclinical trial is able to treat corneal neovascularization more effectively compared to the commercial eye drop even at a lower dosage (Yuan et al. 2015). Durasite® DDS is polycarbophil-based aqueous solution wherein polycarbophil is

a polyacrylic acid attached to divinyl glycol that forms hydrogen bond with the negatively charged mucus, corneal and conjunctival epitheliums, to prolong the effect of the drug. Betoptic S®, marketed in 1990 whose active constituent is betaxolol, is used for glaucoma therapy which is attached to negatively charged sulfonic groups in resin. When the formulation is practiced in the eye, cationic ions like Na + or K+ stimulate the release of the drug from the complex into the tear film and lead to penetration across the cornea. TobraDex® ST is a mixture of tobramycin (0.3%) and dexamethasone (0.05%) which has been used as an anti-inflammatory and anti-infective formulation for blepharitis. The viscosity is increased after mixing with tears for an extended duration, increasing the bioavailability of the drug (Kuno and Fujii 2011). Among nanoparticles, polyethylene glycol (PEG), hyaluronic acid, and chitosan are mainly utilized to promote the residence time of nanoparticles (Bu et al. 2007).

1.4.3 Nanoparticles for Cancer Therapy

Cancer is a severe health problem and is becoming a significant cause of deaths all over the world. The extent of cancer has elevated from 12.7 million in 2008 to 14.1 million declared by the last report of world cancer mentioned by the World Health Organization (Stewart and Wild 2014). The number of cancer cases is expected to increase by 75% per year. There are many chemotherapeutic agents employed for the treatment of cancer like DNA damaging agents, plant alkaloids, antimetabolites, and terpenoids (Sutradhar and Amin 2014). However, these potent agents have specific challenges like lack of target specificity which ultimately results in toxicity and clinical failure, lack of aqueous solubility, and lack of a mechanism of drug resistance in cancer therapy (Wakaskar 2018). The application of nanomaterials can improve the problems of cancer therapeutics. Their optimum size and surface properties are some important characteristics for the nanodrug delivery in cancer therapy through either active targeting or passive targeting. Differently targeted nanomaterials are also available, which proves to be successful in cancer treatment. The therapeutics' delivery using targeted nanomaterials in cancer therapy is beneficial in improving drug/gene delivery via either passive or active targeting. They have shown to increase the intracellular concentration of the therapeutics in cancer cells without any toxicity. The receptor-mediated drug delivery is shown in Fig. 1.6.

Along with this, the targeted nanomaterials can also be developed as a pH-sensitive or temperature-sensitive drug vehicle. The pH-sensitive carrier system can be convenient in delivering drug within the acidic microenvironment of cancerous cells. The temperature sensor can take and deliver the drug according to the changes in temperature in the tumor region through ultrasound waves, magnetic fields, etc. (Saad et al. 2008). It was demonstrated that polyethylene glycol-coated liposome-entrapped soluble prodrugs were discovered to bury the problem of instability. The rat plasma was utilized in in vitro hydrolysis investigation, and they were found to

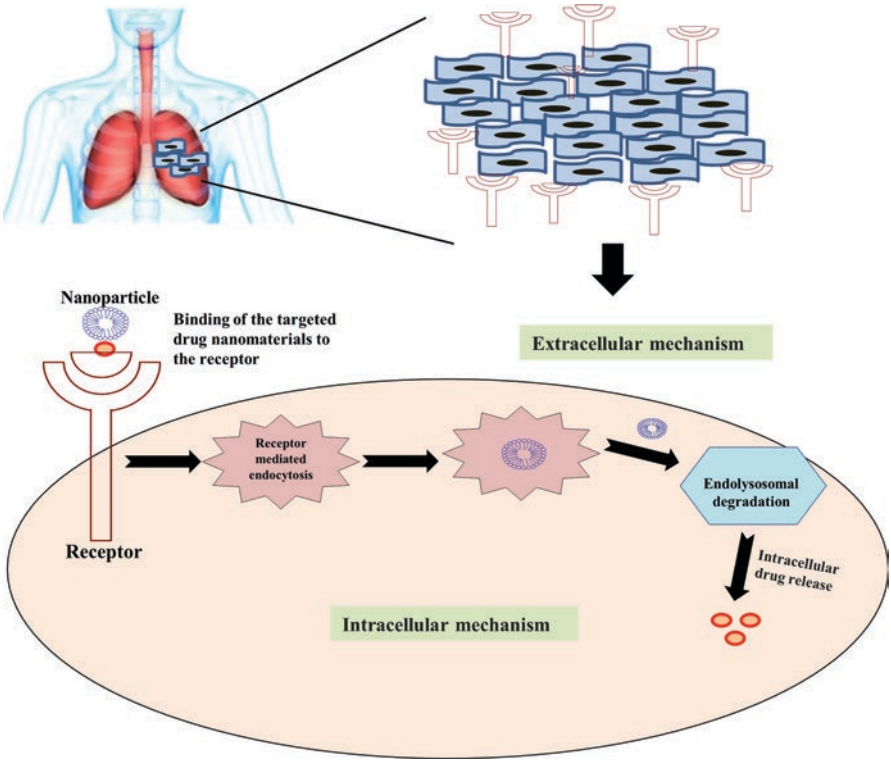

Fig. 1.6 Diagrammatic representation of receptor-mediated drug delivery to lung cancer cells. Drug-nanoparticle combination binds to a receptor on the membrane, interferes with the localization of nanoparticles through endocytosis, and releases drug by lysosomal degradation to the active sites of tumor cells

be entirely stable, and crystallization of paclitaxel was not observed. It has also been demonstrated that hydrophobic doxorubicin can be encapsulated in the shell region of PEG-PLA block copolymers and hydrophilic anti-Bcl-2 siRNA in their core region of the copolymers. The drug/gene co-loading copolymer exhibits high loading efficiency and excellent stability compared to liposomes (Lim et al. 2013a). The iron oxide-based magnetic nanomaterials are considered to be the most auspicious hyperthermia-specific agents. Iron oxide powder was employed for the treatment of metastatic bone tumors and hyperthermia-induced apoptosis in different patients (Matsumine et al. 2007). Photothermal studies of nanocages displayed a photothermal cell damage power density threshold of 1.5 W/cm^2, compared to gold nanoshells (35 W/cm^2) and gold nanorods (10 W/cm^2). The nanocages were attached to the monoclonal antibodies (anti-HER2), which can be used to target epidermal growth factor receptors (EGFRs) that are overexpressed on the surface of breast tumor cells. The results demonstrated that shape- and size-regulated gold nanomaterials could be potential for tumor therapy (Jingyi Chen et al. 2007). Presently, there are

some nanomaterial therapeutics, imaging agents, and technologies which are clinically approved. For example, VYEXOS/CPX-351 is a combination of the synergistic ratio of two anticancer drugs, and early clinical results have interpreted the suggested dose with survival advantages displayed in some patients as compared to standard chemotherapy regimens. Camptothecin, a highly toxic drug investigated in a cyclodextrin nanoparticle (CRLX101), results in tumor reduction in 74% of patients (Svenson et al. 2011). Similarly, LiPlaCis (Cisplatin encapsulated liposomal formulation) (de Jonge et al. 2010), NC-6004 Nanoplatin (Micelles containing cisplatin) (Plummer et al. 2011), and NC-4016 DACH-Platin (Polymeric micellar nanoparticles containing diaminocyclohexane platinum) are some examples of encapsulated platinum-based chemotherapy nanoformulations that are extremely successful and already approved.

1.5 Challenges of Nanomaterial for Drug Delivery

There are many challenges in the field of nanotechnology during drug delivery. The most critical issues in antitumor therapy are toxicity of the nanomaterials (Kostarelos et al. 2009). These reported nanomaterials have potential risks and challenges. The toxicity of the nanomaterials like quantum dots cannot be ignored as they carry few heavy metals. Some nanomaterials may be linked to the surface of the cellular membrane through adsorption or electrostatic interactions and destroy the cells through the production of reactive oxygen species, which lead to cell death (Hoshino et al. 2011). Sometimes, cancerous cells develop drug resistance over a particular drug treatment, making released drug ineffective. Combinatorial therapies like the adoption of targeted nanomaterials for the delivery of therapeutics, like gene as well as anticancer drugs, might be adequately distributed and target cancerous cells and tissues to overcome drug resistance and block tumor growth. Development of multifunctional targeted nanomaterials could be another approach which can be employed to overcome drug resistance problem. Another challenge of targeted nanomaterials is that nanomaterials might change the solubility, stability, and pharmacokinetic behavior of drugs. But these characteristics have not been broadly explored. The toxicity, aggregation problem, and shelf life of the component used for developing nanomaterials are various challenges for their use. However, some properties of nanomaterials like poly(lactic-co-glycolic acid) are not distributed in tissues for a prolonged duration and have low toxicity, but deteriorate rapidly, which might be beneficial for drug\gene delivery. Other nanomaterials like carbon nanotubes and quantum dots are strong and can remain in the body for several weeks or may be years, resulting in limited use for repeated utilization in treatments. The silicon/silica has been gradually taken out due to possible health issues (Jain et al. 2011). It was also reported that carbon nanotubes could penetrate the central nervous system through the blood-brain barrier which may cause different types of diseases. The toxicity of CdTe/CdS quantum dots was reported in in vitro and in vivo experiments. The quantum dot-influenced toxicity was noticed in the cell

lines along with autophagy. In a murine model, there is splenic injury, liver damage, hematopoietic disturbance, and nephrotoxicity, caused upon injecting quantum dots (Fan et al. 2016). The toxicity of some metal nanoparticles generally depends on the size and surface charge. Not all metal nanoparticles have toxicity (Sharma et al. 2018). Other parameters like particle size, drug encapsulation, shape, distribution in the body system, and cost need to be appropriately addressed for better development of nanomaterials. The optimal selection of the right nanomaterials and particle size is an important aspect in cancer therapy, as the elimination rate of small particle-sized nanomaterials is high and mostly deposited in the liver and spleen, making it inadequate, because large-sized particles may be too large to go through small capillaries for drug delivery. Several reports showed that reactive oxygen species production takes place in different metal nanoparticles like titanium oxide, zinc oxide, iron oxide, and aluminum oxide. The toxicity of nanoparticles can be reduced by coating them with different polymers. For example, the toxicity of zinc oxide nanoparticles can be minimized by surface functionalization with silica coating. Silica coating prevents dissociation of zinc nanoparticles to zinc ions (Roa et al. 2012). In case of ocular drug delivery, various challenges still exist in future studies which include less number of in vivo studies in numerous ocular disorder therapies. More efforts are required in ocular drug delivery as animal models especially ocular cancers should be well-established. However, the most commonly used animal is a rabbit model because of its high surface sensitivity, high production of mucus, low tear formation, similar size of the human eye, and low frequency of blinking eye. Such differentiation results in better bio-adhesion and holding in the ocular surface, which leads to false results. Biomarkers are the types of targets used for targeted delivery, however, biomarkers related to ocular diseases need to be understood along with their cellular mechanisms and functionality. The slow progress of targeted nanomaterials has been due to a shortage of knowledge regarding circulation and localization of nanomaterials after oral or intravenous administration. Several studies are still not able to investigate the efficacy of the targeted nanomaterials in real time in vivo, which leads to a lack of precise information related to the circulation and therapeutic effects of nanomaterials. Hence, detecting cancer cells in the body and treatment of these cancer cells is also a challenging task, which needs to be addressed for effective targeted nanomaterials.

1.6 Conclusion

The unique properties of nanomaterials like small size and easy surface modification enable it to be applicable in the field of biomedical sciences. It improves the bioavailability, poor solubility, extreme toxicity, and lack of selectivity of the drug to the desired targets. It helps in eliminating the complications of problematic drug or potential plant-based therapeutic molecules. In addition to the significant advantages, there are a few disadvantages associated with the nanomaterial formulation, physical handling difficulties, or aggregation. Hence, it is important to develop

advanced technological methods which may lead to the improvement in quality. Despite a few drawbacks, nanomaterials have some functional groups on its surface which allow the different drugs to conjugate in an appropriate ratio. Well-designed nanomaterials show potential to penetrate the tumor either through passive or active targeting and ultimately increase the cytotoxic effects of the agents. There are several nanoparticles reported which play a key role in the drug delivery system. The nanoparticles also showed potential in carrying and delivering the drug to the target site, are used in fluorescent imaging and sensors, and sometimes have inherent therapeutic activity. Many anticancer drug-loaded nanoformulations are already USFDA approved, and some are in clinical trials. Only a few of the drug-loaded nanoparticles are available in the market like Doxil® (doxorubicin) or DaunoXome® (daunorubicin). The role and scope of nanomaterials for drug delivery in biomedical science are increasing, and the production of advanced, efficient, and multifunctional nanomaterials will not be so far in the upcoming future. The drug delivery system through nanomaterials helps in resolving the problems of targeted drug delivery. However, before a successful clinical phase, there is a need for further extensive research in the field of nanotherapy, and efforts are under way worldwide to exploit the technology for better health care.

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Chapter 2
Nanocarriers as Potential Targeted Drug Delivery for Cancer Therapy

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Abstract Cancer is a disease characterized by the uncontrolled growth of cells and is the leading cause of death worldwide with an incidence of 11 million new cases each year. Nanotechnology-based drug delivery systems have received much attention for cancer treatment. Nanocarriers are the delivery systems which are prepared by alteration of the size (1–1000 nm) and shape of a material to the nano-range level. Nanocarriers are prepared by utilizing natural, polymeric, inorganic magnetic silica-based materials. Various nanocarriers including liposomes, solid lipid nanoparticles, polymeric nanoparticles, dendrimers, magnetic nanoparticles, and other inorganic nanoparticles have been investigated for diagnostic, therapeutic, and drug targeting in cancer therapy. Nanocarriers act as a cancer-specific drug delivery or diagnostic agent by inherent passive targeting mechanism or adopted active targeting strategies by altering the surface properties with specific ligands. Targeted nanoparticulate systems increase the accumulation of the chemotherapeutic agent in the tumor tissue and reduce the toxicity to healthy cells. Nanocarriers extend the drug release for a longer duration and protect the drug from degradation. Nanocarriers are also proven effective for improving the pharmacokinetics of poorly soluble hydrophobic drugs by solubilizing or permeating them through lipophilic biological barriers.

Approximately 1500 patents are filed with respect to nanocarrier-based formulation of cancer therapeutics. However, clinically approved nanocarrier-based therapeutics are very few in number, but the trend reveals the number of nanocarrier-based formulations is increased in recent years. The clinical studies of nanocarrier-based formulations have shown improved safety and efficacy. The main hitch in the commercialization of nanocarriers is the difficulty to achieve optimum particle size distribution, scale-up of the formulation, and reproducibility. Conversely, nanocarrier-based therapeutics lack adequate guidelines from drug regulatory authorities. The proposed chapter will address the different nanocarriers and advances in the surface engineering of nanoparticles for cancer cell targeting, diagnosis, and drug delivery applications. The focus of this book chapter is to provide an insight into various nanocarriers for their multiple applications in the treatment of cancer.

Keywords Cancer · Chemotherapeutic agent · Cytotoxicity · Drug delivery systems · Multidrug resistance · Nanocarriers · Nanotechnology · Nucleic acid · Reticuloendothelial system · Targeted drug delivery · Tumor

Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
EPR	Enhanced permeation and retention effect
DSPE	Distearoylglycerophosphoethanolamine
DOPC	Dioleoyl phosphatidylcholine
RES	Reticuloendothelial system
PEG	Polyethylene glycol
DPPC	Dipalmitoyl phosphatidylcholine
MPPC	1-Myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine
PLGA	Poly(lactide-co-glycolide)
PBEMA	Phenylboronic ester-functionalized methacrylate

2.1 Introduction

Cancer refers to an uncontrolled growth and division of cells with the potential to invade or spread to other parts of the body. In contrast to benign tumors, cancerous tumor is characterized by abnormal proliferation and uncontrolled growth of cells which leads to fast spreading in the body. Invasion and metastasis are two common processes which basically involve healthy adjacent tissue and distant tissue through lymphatic transportation or the bloodstream. After chronic metabolic ailments, cancer is the leading cause of annual deaths worldwide with an incidence of 11 million new cases each year (Nci 2019). Among the different types of cancer, skin cancer holds the top of the charts followed by breast and prostate cancer in women and men (Bregoli et al. 2016). The treatment is usually based on a combination of chemotherapy and/or radiation with surgical excision of the tumor (Williams et al. 2008).

Research on cancer is based on devising newer drug delivery strategies which can specifically target cancer cells. Significant progress has been achieved in the development of newer agents that are effective against cancer. These approaches are based on the identification of newer targets for cancer and devising active strategies for cancer. This chapter has discussed nanotechnology-derived drug delivery systems in cancer chemotherapy. In the beginning an overview of cancer drug therapy with emerging therapeutic approaches is discussed, followed by overcoming the barriers including physiologic, pharmacokinetic, and physiochemical of the tumor cells. Advanced drug delivery strategies are improvised to breach these barriers and are presented along with examples in this chapter. However, most of the research is in preclinical stages, and formulation scientists have successfully optimized the therapeutic outcome across various cancer types. This chapter will give insight into the future of cancer therapy, challenges faced, and clinically effective formulations for treating cancer malignancy (Williams et al. 2008).

2.2 Conventional Strategy for Cancer Treatment

Presently, chemotherapy, surgical excision, and radiation therapy are mostly used for the treatment of cancer. Each technique comes with its own adverse effects and limitations. However, physicians usually prefer the surgical removal of the tumor as the first option (Harrington et al. 2010; Kypriotakis et al. 2016). Also, surgical excision is well known to be ineffective except in large tumors, as the cancer cells do infiltrate nearby vital organs or show signs of distant metastasis. Further, cryosurgery is another option where freezing the tumor cells induces degeneration. This is a substitute for excision which is mostly advantageous to prevent distant metastasis and to eradicate non-cancerous or precancerous lesions. Commercially available antineoplastic drugs have been used as chemotherapy for limiting cellular proliferation. Chemotherapy may be intravenous injections, oral dosages, topical, or installable implant. The lysis of healthy tissue (cytotoxicity) has always been an issue of concern with conventional dosage forms of antineoplastic agents in use. The prominence of cytotoxicity is well observed in rapidly dividing cells such as cells of gonads, hair follicles, digestive tract, and bone marrow, thereby causing side effects and associated disorders which include vomiting, nausea, alopecia, infertility, immunosuppression, anemia, thrombocytopenia, and leukopenia (Bacci et al. 2004; Mandalà and Tondini 2012; Singhvi et al. 2018b). Apart from chemotherapy, radiation therapy is also very popular and the preferred technique which leads to the destruction of cancer cells and reduction of the tumor mass by focusing the radiation energy (Durrant and Scholefield 2003). Blood stem cells and bone marrow transplantation may be used as an assistive measure to combat cytotoxicity issues due to high doses of radiation and chemotherapy. Other techniques may involve immunotherapy which is synonymously used for biotherapy as it exaggerates the human body's immune system to destroy cancerous cells. The response is stimulated through the induction of interleukins, vaccines, monoclonal antibodies, gene therapy, colony-stimulating factors, or immunomodulators (non-specific). Gene therapy is a pioneer among the cancer study as a future perspective toward cancer treatment (El-Aneed 2004). This involves transferring genetic material into the cancer cells to induce lysis. Some of the inhibitors for angiogenesis are under investigation and under evaluation in clinical trials. The development of newer blood vessels is an important factor responsible for the growth and spread of cancer. This provides an opportunity to cancer cells as a source of oxygen and nutrients, thus spreading to different parts of the body (Fayette et al. 2005; Fernandez-Fernandez et al. 2011). These angiogenesis inhibitors basically prevent the formation of newer blood vessels, thus depriving the cancer cells of nourishment and ultimately leading to cellular death. Also, the hyperthermia technique is well established nowadays, where the lysis of cancer cells is caused by inducing higher temperatures and availing minimal injury to normal cells (Kumar and Mohammad 2011). The mechanism involved is damaging the protein structure and thus cellular degradation (Johannsen et al. 2007). An added advantage of this hyperthermia is it may exacerbate the radiation-related killing of cancer cells especially the one which is less prone to the radiations (Jha et al. 2016). Hyperthermia may be induced by high-

intensity laser beams (Pathak and Thassu 2009). The laser can also be involved as it may cause shrinking or destruction of the tumor. This can be widely used to suppress the superficial tumor destruction and lining of internal organs. Photodynamic treatment therapy is another approach which utilizes a photosensitizer drug or agent (Robertson et al. 2009). Photosensitizers when exposed to a specific wavelength produce singlet oxygen species which may destroy the cancer cells. Targeting approach may be utilized so that specific drugs may be used to destroy the tumor cells and block metastasis. The scientific community has already shifted to nanotechnology-based carrier systems which can target the drug at a sharpened rate, resulting in better destruction and reduced toxicity (Strausberg et al. 2004).

2.3 Mechanism of Action of Chemotherapeutic Agents

Based on the mechanism of action, antineoplastic agents are divided into three major categories as shown in Fig. 2.1 (Pathak and Thassu 2009; HERTZ et al. 2015).

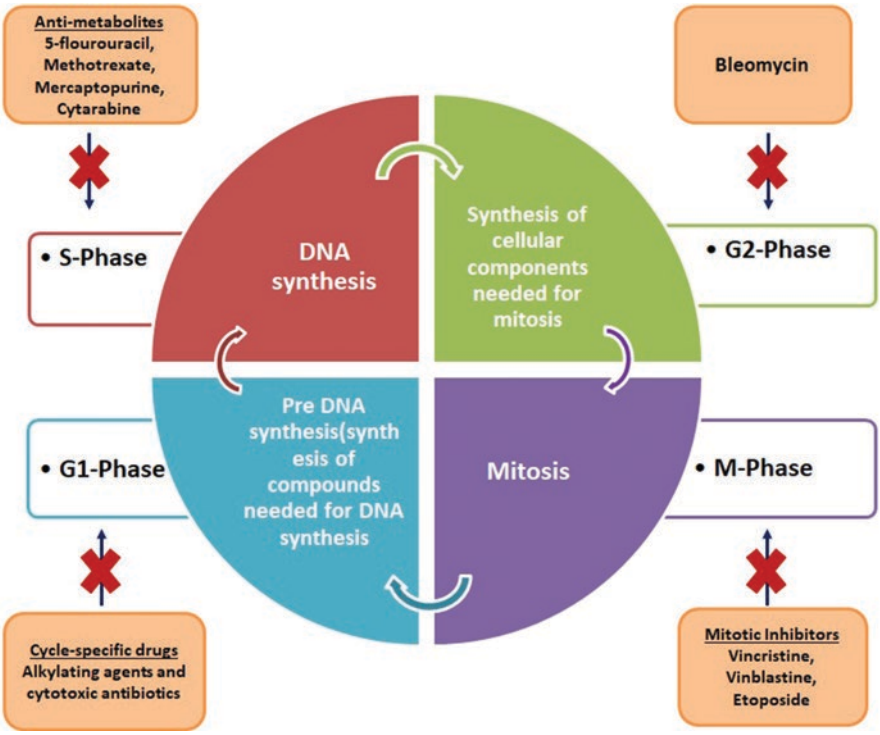

Fig. 2.1 Cell cycle and point of action of phase-specific drugs. Schematic representation of the cell life cycle with the series of events during cell division. Different classes of therapeutic agents acting in different phases of the cell cycle inhibiting cell division

2.3.1 Pre-Deoxyribonucleic Acid Block Synthesis Prevention

Building blocks of deoxyribonucleic acid (DNA) including nucleotides and heterocyclic bases are synthesized in vivo. Chemotherapeutic agents under this category block the few steps in the de novo synthesis of nucleotides, thereby restricting the synthesis of DNA and ribonucleic acid (RNA) and finally limiting cellular replication. This impairment in DNA synthesis obscures cellular proliferation (Huehls et al. 2016). The drugs of this kind are methotrexate, 5-fluorouracil, mercaptopurine, and hydroxyureas.

2.3.2 Chemical Damage to Cellular Deoxyribonucleic Acid

Few agents destroy the DNA and RNA of cancerous cells. DNA and RNA replication is halted totally and inhibits cancer cell formation (Yang et al. 2014). The drugs in this class comprise of cisplatin and antibiotics like doxorubicin, daunorubicin, and etoposide.

2.3.3 Disruption of Mitotic Spindle Synthesis

Mitotic spindles align themselves to form railroads in the form of the North and South Pole. These do split up at the time of division (Woo et al. 2015). Drugs including paclitaxel and vinca alkaloids disrupt spindle formation and thereby cellular division.

2.4 Barriers and Obstacles to Drug Delivery to Cancer Cells

Despite the level of progress in cancer research and drug development, there still exist loopholes and barriers in the development of systemic delivery of drugs to cancer cells. These include physicochemical and physiological barriers. Drug delivery scientists are trying hard to devise a novel strategy to surpass the obstacles, in concern to provide treatment therapies to cancer cells only with minimum side effects (Williams et al. 2008). The barriers are summarized in Table 2.1.

Table 2.1 Physiological barriers at various levels

Physiological barriers		
At tumor level	At the cellular level	Other barriers
Increased interstitial fluid pressure	Multidrug resistance through adenosine triphosphate-binding cassette transporters	Physicochemical barriers (solubility, pKa, lipophilicity)
Hypoxic states	Stress-mediated	Blood-brain barrier
Reduction in microenvironment pH	Resistance to apoptosis	Intestinal absorption

2.4.1 Physiologic Barriers

Drug delivery aims to efficiently deliver the drug by overcoming the physiological barrier both at the cellular level and tumor level (Vijan et al. 2012). Systemic delivery of drugs becomes limited when organs such as the central nervous system and gastrointestinal system are concerned. These barriers comprise of:

Barriers in Physiology at Tumor Level

Various literature findings have explored and outlined these barriers (Cairns et al. 2006; Folkman 2008; Danhier et al. 2010). The major barrier to this is the malformed vasculature and impaired lymphatic system that collectively engages as a microenvironment which is a barrier to cancer chemotherapy. The major representatives of the tumor microenvironment are:

Increased Fluid Pressure in the Interstitium

Combination of leaky vasculature and poor lymphatic drainage leads to enhanced permeation and retention (EPR) effect, which is a deciding factor to high molecular weight compounds. This also stands true for increased retention of plasma proteins and thus rise in interstitial osmotic pressure and thereby swelling (Jang et al. 2003). Also, this increased interstitial fluid pressure (IFP) leads to convective fluid flow from the center of the tumor and thus limits the drug transport in the tumor tissue.

Hypoxic Microenvironment

A reduction in oxygen concentration due to increased oxygen consumption with impaired blood flow leads to hypoxic and acidic regions inside the tumor. Oxygen is a potent radiosensitizer, and a decreased response to radiotherapy is seen. This is accompanied by a decreased efficacy of chemotherapeutic agents (Cairns et al. 2006).

Reduced Extracellular pH

The reduction in extracellular pH compared to surrounding tissue is due to impairment in the drainage of metabolic end products from the interstitium of the tumor. The acidic environment limits the permeability of organic cationic chemotherapeutic agents. At lower pH values, ionization is observed, leading to reduction in cellular uptake of these cations (Williams et al. 2008).

Barriers in Physiology at the Cellular Level

Cellular resistance to drug therapy is induced due to alteration in the biochemical aspects of cancer cells. Different pathways are responsible for this including the following:

Resistance to Multidrug by Adenosine Triphosphate-Binding Cassette Transporters

Tumor cells have specialized membrane proteins that catalyze the transport of drug and limit intracellular drug concentration. Chemotherapy faces a major setback due to multidrug resistance (MDR) (Tsuruo et al. 2003; Jabr-Milane et al. 2008). MDR is the development of cellular resistance to a broad range of structurally and functionally unrelated compounds (Thomas and Coley 2003). This resistance is generally produced in response to a single substance. A plasma protein was identified to be overexpressed in the colchicine-resistant tumor cells by Luliano and Ling in 1976. This protein was identified as P-glycoprotein (P-gp). These P-glycoproteins are a subset of adenosine triphosphate-binding cassette (ABC) superfamily (Kathawala et al. 2015; Jones and George 2015). Numerous members from this family have now been found out to be associated with multidrug resistance in human cancer cells including P-gp multidrug resistance-associated proteins and breast cancer resistance protein (Breedveld et al. 2006; Holohan et al. 2013). Reversal of multidrug resistance is under trial by inhibiting the transporters to improvise the efficacy of cancer chemotherapy.

Stress-Mediated Resistance

Barriers in the physiology of the tumor microenvironment (reduced pH and hypoxia) cause glucose-regulated stress response to cancer cells. This induces resistance to multiple chemotherapeutic agents and drugs. The specific mechanism is also involved in the reduction of resistance including that of decreased expression of DNA topoisomerase by a few drugs like topotecan and etoposide. The issue with this is reversible of nature and dissipation of response when stress conditions are removed (Tsuruo et al. 2003; Tredan et al. 2007).

Resistance to Apoptosis

Cancer therapy aims at initiating the tumor-programmed death or apoptosis. Chemotherapeutic agents basically induce apoptosis in tumor cells, and resistance is attained by disruption of this machinery by the cancer cells. Increased expression of glyoxalase I is a marker to apoptosis resistance in cells, thereby a potential target for reversing the phenomenon (Tsuruo et al. 2003).

2.4.2 Other Barriers to Physiology

For the efficient delivery of drugs, the physiological obstacles that act as barriers must be overcome. When a chemotherapeutic agent is targeted for brain cancer, the blood-brain barrier stays as an obstacle in delivering drugs to the central nervous system (Begley 2004). This is because of the active efflux mechanism of these agents that reduces the uptake of drugs in the brain. Members of the adenosine triphosphate-binding cassette family of transporters like breast cancer resistance protein and P-glycoprotein show such activity. Thus, there is a need to inhibit these transporters to enhance the central nervous system penetration of such drugs. By knowing the nature and mechanism of physiological barriers that decrease the drug uptake, one can develop alternatives to improve the absorption. One such example is the delivery of proteins and peptides that are degraded by gastrointestinal fluids because of their instability. Thus, coating or encapsulation can avoid their contact with these fluids and prevent the drug's degradation. Efflux transporter system in the intestine limits the uptake of nutrients and drugs administered orally. This leads to decrease in the bioavailability of drugs administered orally. Inhibition of these transporters may improve the bioavailability of ingested drugs (Williams et al. 2008).

2.4.3 Physicochemical Properties of Compound Acting as Barriers

Physicochemical properties of drugs are also responsible for the alteration of their effectiveness against cancer cell targeting. The molecular weight of the compound, its chemical structure, and its dissociation constant are important parameters to be considered for the delivery of drugs into tumor cells. P-glycoprotein substrates and the transporters are defenseless against multidrug resistance and show restricted transport across the gastrointestinal tract and blood-brain barrier.

Solubility in aqueous conditions holds an important aspect of effective drug therapy. Chemotherapeutic agents belonging to taxanes have poor solubility. The improvement in solubility can be achieved by the addition of nonionic surfactants to solubilize the drug. These agents include Cremophor EL and Tween 80 (Thomas

and Coley 2003). Unfortunately, these are pharmacologically active solubilizers and are associated with hypersensitivity, peripheral neurotoxicity, and dyslipidemia condition. These drawbacks have led to the development of alternative delivery systems for poorly soluble agents (Nobili et al. 2009). Advancement in nanotechnology has opened the door for advanced drug delivery systems to improve bioavailability and targeting cancer cells to improve the efficacy of drug candidates with minimum toxicity effects to normal cells.

2.5 Nanocarrier Drug Delivery for Cancer Therapy

Early diagnosis at the initial stages of carcinogenesis is the major step in cancer treatment. Numerous scientific studies have explored new, innovative, and noninvasive tools for such purposes. Quantum dots, cantilevers of nanoscale, have been proven to be the tools for cellular-level identification of cancer (Wee et al. 2005). The treatment aims at eradicating fully developed cancer cells without harming normal healthy cells of the body (Wegner and Hildebrandt 2015; Chandan et al. 2018). This is primarily an option to reduce tumor invasiveness. Nanotech-based approaches have been tried out to formulate nanoparticles as potential carriers of anticarcinogenic agents. These include polymeric nanoparticles, polymeric micelles, magnetic nanocarriers, ceramic nanoparticles, lipidic nanoparticles, nanoemulsions, nanoparticle composites, dendrimers, nanocapsules, and nanovesicles (Fig. 2.2). Applications evolve in the field of cancer therapy in combination with that of radiation, ultrasound, photodynamic, thermotherapy, and gene delivery mediated by nanoparticles (Jain 2005; Lu et al. 2007; Sun et al. 2008; Gao et al. 2009; Girdhar et al. 2018).

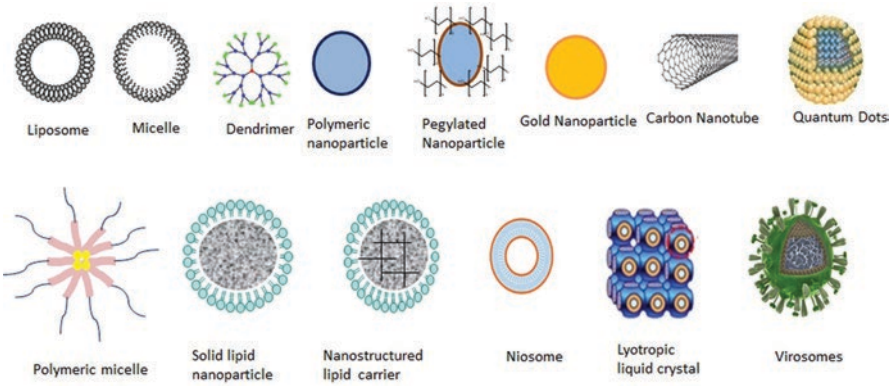

Fig. 2.2 Various nanocarrier systems for delivery of anticancer agents to the tumor cells. These include liposomes, polymeric micelles, dendrimers, polymeric nanoparticles, PEGylated nanoparticles, gold nanoparticles, lipidic nanoparticles, niosomes, and virosomes

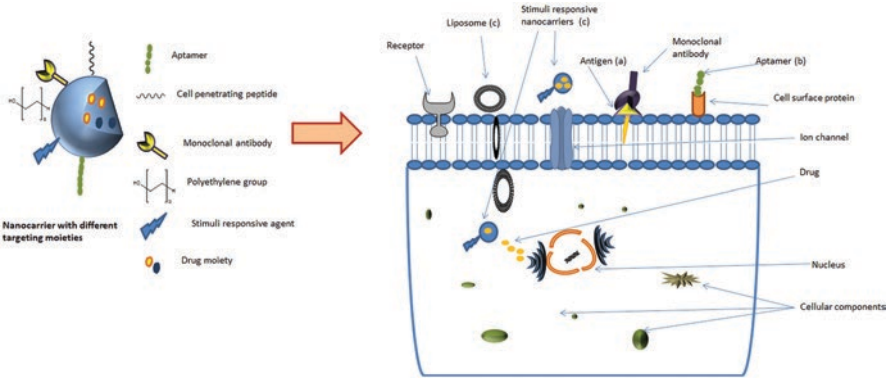

Fig. 2.3 Nanocarrier surface modification with targeting moieties improves the opportunity of targeting. Tumor-specific antigens on the surface of nanoparticles help in the localization of delivery system to targeted cancerous cells. The figure represents surface modified with targeting moieties like aptamer, cell-penetrating peptides, monoclonal antibody, polyethylene glycol, and stimuli-responsive group (pH or temperature sensitive) for targeting drug-loaded nanocarriers to cancer cells

Tumor-specific antigens are identified by impregnating specific antibodies, sugars, peptides, antineoplastic drugs, and hormones on the surface of nanoparticles which help in the localization of the delivery system in the tumor. Surface modification of nanocarriers with targeting moieties improves the opportunity of targeting as shown in Fig. 2.3. Also, there is an improvement in the drug release to the specific receptor sites of cancer cells. This leads to reduction of cytotoxicity effects in healthy cells (Dinauer et al. 2005). Encapsulation of anticancer agents into nanocarriers provides advantage of the reduction in the degradation of encapsulated drug from external physiological environments. Being smaller in size, the penetration of nanoparticles is enhanced such that it can pass through the smallest capillaries too and taken up passively by cancer cells (Pandey et al. 2005). Sustained release can be attained by using biodegradable nanoparticles over a period of time (Cai et al. 2015). Following various nanocarriers, delivery systems have been discussed to understand their unique nature and suitability for the delivery of anticancer agents.

2.5.1 Liposomes

Liposomes are vesicles composed of a bilayer amphipathic lipid molecule enclosing an aqueous compartment. Liposomes constitute of phospholipids and cholesterol. Phospholipids form a bilayer membrane in the aqueous phase and cholesterol acts as fluidity buffer which adds rigidity to liposomes and prevents the drug from leaching out of liposomes. Surfactants or stabilizers such as polyvinyl alcohol (PVA) and poloxamers (pluronics) are added to stabilize the vesicles and prevent aggregation. Phospholipids including distearoylglycerophosphoethanolamine

(DSPE), dioleoyl phosphatidylcholine (DOPC), and dioleoyl phosphatidylglycerol are widely investigated for liposome preparation. Liposomes can encapsulate both lipophilic and hydrophilic drugs, where the internal aqueous environment of the vesicle is suitable for hydrophilic drug and the lipid bilayer is suitable for lipophilic drug entrapment. Liposomes are mostly versatile nanocarriers used for entrapment of drugs due to their biocompatibility, non-immunogenic nature, and biodegradability which made it the most favorable nanocarriers for cancer drug delivery. Liposomes are mainly classified as unilamellar and multilamellar vesicles. Liposomes are colloidal carriers due to their highly lipophilic nature and they are prone to reticuloendothelial system (RES) clearance by macrophages localized in the spleen and liver (a process called opsonization). This localization is useful in passive targeting for liver and spleen cancer. To overcome opsonization, surface modifications can be done on nanocarriers with hydrophilic molecule attachment which is termed as stealth nanocarriers (stealth liposomes). Doxil® is a Food and Drug Administration-approved PEGylated liposome (PEG [polyethylene glycol]) with prolonged blood circulation half-life compared to normal liposomes. To improve the efficacy of liposomes, various modifications can be done on liposomes apart from PEGylation (Barenholz 2012; Jain and Jain 2018).

Liposomes are found to be advantageous in the delivery of both hydrophilic and lipophilic drugs; however, nonselective and ineffective tumor targeting limited the utilization of liposomes. To overcome these limitations, liposomes are further modified as pH-sensitive, thermo-responsive, redox-responsive, light-responsive, enzyme-responsive, and magneto-responsive for targeting and selective drug release at the tumor environment.

The pH-sensitive liposomes are more efficient in delivering drugs with pH-sensitive composition. In cancer cells, continuous generation of energy from anaerobic glycolytic metabolism of glucose leads to increase in lactic acid level. This increased lactic acid leads to decreases in the pH of the tumor environment compared to the normal extracellular environment or blood pH (7.4). For the preparation of pH-sensitive liposomes, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine is widely used in combination with oleic acid and cholesteryl hemisuccinate. Cholesteryl hemisuccinate is an amphiphilic molecule which gets protonated at acidic pH. This combination forms liposome at neutral pH and gets destabilized at an acidic environment triggering the release of the drug at the tumor site (Kanamala et al. 2016; Heidarli et al. 2017). Apart from pH-sensitive lipids, liposome surfaces can be further modified by fusogenic peptides like hemagglutinin which is derived from virus and listeriolysin O from bacteria or by utilizing surfactants (Varkouhi et al. 2011). Some acid-responsive polymers like N-isopropylacrylamide, poly(glycidol)s, and poly(alkyl acrylic acids) are investigated for pH-sensitive liposome preparation (Yoshizaki et al. 2014; Naziris et al. 2017).

Hyperthermia is the thermal therapy used in cancer treatment where body tissue (tumor) is exposed to a higher temperature (113 °F) which can damage cancer cells with minimal damage to normal tissue. It is mostly preferred in superficial tumors. Due to the lack of precise thermometry and inability to target deep tumors, hyperthermia treatment is not preferred in cancer treatment. But the combination of

hyperthermia or radiation with chemotherapy has shown improved efficacy in many studies (Cihoric et al. 2015). This improved efficacy might be results of (i) selective accumulation of liposomes at the target site due to its increased vascular permeability and (ii) triggered the release of drugs from a thermosensitive carrier within the tumor vasculature and (iii) interstitium (Manzoor et al. 2012; Ta and Porter 2013).

Affram et al. developed a thermosensitive liposomal nanoformulation of poorly permeable drug gemcitabine and gadolinium to target the solid pancreatic tumor with an aid of local mild hyperthermia. Thermosensitive liposomes of gadolinium (Magnevist®) were prepared to increase magnetic resonance imaging contrast in solid tumor. Compositions of liposomes including dipalmitoyl phosphatidylcholine (DPPC), 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine (MPPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol) 2000 (DSPE-PEG_{2000}) were used at molar ratio of 90:10:4 in case of gemcitabine. DPPC, MPPC, DSPE-PEG_{2000}, and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (gadolinium salt) (Gd-DSPE) were used at molar concentration of 70:5:4:20 in case of gadolinium. In vivo studies performed in the MiaPaCa-2 tumor model revealed inhibition of tumor growth by increased accumulation of thermosensitive liposomes up to 3.5-fold higher in comparison to free gemcitabine. A similar trend was observed in the case of gadolinium, and this study reveals targeting drug delivery utilizing thermosensitive liposomes (Affram et al. 2017).

2.5.2 Niosomes

Niosomes are bilayer amphiphatic microvesicle nanocarriers similar to liposomes, in which phospholipids are replaced with nonionic surfactants. Niosomes can entrap hydrophilic, amphiphilic, and lipophilic molecules and can exhibit longer circulation time compared to liposomes. Surfactants including polyoxyethylene 4 lauryl ether (Brij 30), polyoxyethylene stearyl ethers (Brij 72 and 76), and sorbitan fatty acid esters are mostly used in niosomal formulations (Kazi et al. 2010; Kumar and Rajeshwarrao 2011). Niosomes are delivery systems which attenuate disadvantages of liposomal nanocarriers like instability, purity related to phospholipids, and the high cost of phospholipids. Nonionic surfactants of niosomal vesicles can improve the bioavailability of poorly soluble drugs. These vesicles are found to be P-glycoprotein inhibitors which can be used to improve cellular uptake of drugs. Modification in niosomal vesicle by incorporating targeting moiety can be utilized for delivery of anticancer drugs to cancer cells. Targeting moieties like magnetic material encapsulated along with the drug molecule and hyaluronic acid composition in niosomes have been proved to increase localization in tumor cells (Tavano et al. 2013; Kong et al. 2013).

Alemi and coworkers prepared PEGylated niosomes in a combination of curcumin and paclitaxel. The particle size of the optimized formulation was up to 90 nm with high entrapment efficiency. The formulation exhibited desired stability,

and in vitro studies revealed enhanced cellular uptake in the MCF-7 cell line (Alemi et al. 2018).

Curcumin- and doxorubicin-loaded PEGylated niosomes were investigated for its selective delivery to cancer cells. In this study, the niosome surface was modified with CGNKRTR (tLyP-1) homing peptide. tLyP-1 peptide penetrates through neuropilin-1 (NRP-1), a transmembrane protein which is overexpressed on the surface of both glioma and endothelial cells of angiogenic blood vessels. In vitro studies performed on human glioblastoma (U87) and human mesenchymal stem cells (hMSC) revealed co-administration of curcumin and doxorubicin significantly improved the anti-glioma effect (Ag Seleci et al. 2017).

2.5.3 Transfersomes

Transfersomes are elastically deformable vesicles similar to liposomes. Edge activators are incorporated to the lipid bilayer to generate elasticity which helps in penetration enhancement through the skin barrier. Surfactants are used as edge activators to destabilize the bilayer vesicles and improve the permeability of the skin lipid layer. Sodium cholate, Span 80, Tween 80, and glycyrrhizinate are commonly used surfactants or edge activators in transfersome formulations. Transfersomes have high potential to penetrate the skin for delivery of drugs; hence, they can be preferred for topical drug delivery for melanoma (skin cancer). 5-Fluorouracil was delivered as transfersomes which are found to be better in comparison to standard liposomes in skin delivery (Estanqueiro et al. 2015).

Jiang and his coworkers formulated an oligopeptide hydrogel containing paclitaxel encapsulated in transfersomes, where the surface was modified with cell-penetrating peptides to treat melanoma. In vivo studies were performed in the xenograft B10F16 melanoma mouse model. The cell-penetrating peptide exhibited improved permeation through the skin and tumor stroma. The mouse treated with paclitaxel transfersomes along with systemic Taxol showed effective reduction of melanoma (Jiang et al. 2018).

Kong and his colleagues evaluated the targeted lymphatic drug delivery of doxorubicin via transdermal route utilizing transfersomes. The transfersome surface was modified with hyaluronic acid which was linked with glyceryl-α-monostearate. In vitro studies demonstrated three times improved accumulation of doxorubicin-loaded transfersomes in comparison to free solution. In vivo studies revealed that the transdermal delivery of hyaluronic modified transfersomes showed significant accumulation in the lymphatic system. In comparison to normal transfersome, hyaluronic-glyceryl-α-monostearate modified transfersomes exhibited 9 times higher cellular uptake in endocytosis of breast tumor cells (MCF-7) (Kong et al. 2015).

2.5.4 Ethosomes

Ethosomes are modified liposomes in which ethanol is used to enhance the permeation of vesicles. Ethanol in ethosomes enhances the permeability of vesicles through the skin by disturbing the skin's lipid bilayer. Paclitaxel was delivered as ethosomes and increased skin permeation was found in squamous cell carcinoma (Estanqueiro et al. 2015).

Paolino and team formulated paclitaxel-loaded ethosomes for topical application in squamous cell carcinoma. In vitro studies were performed in the stratum corneum epidermis model which showed improved permeation of paclitaxel-loaded ethosomes and increased antiproliferative activity in a squamous cell carcinoma model compared to the free drug. This study revealed that ethosomes can be utilized as a potential drug delivery system to treat squamous cell carcinoma (Paolino et al. 2012).

Eskolaky and coworkers evaluated the efficacy of PEGylated ethosomal formulation of paclitaxel. The mean diameter of PEGylated ethosomes was 138.1 ± 2.7 nm, with −13.1 mV zeta potential, whereas the entrapment efficiency was found to be 96 ± 1.27% with 2.82 ± 0.27% drug loading. The in vitro toxicology studies of transfersomes exhibited 4.5-fold increased cytotoxicity compared to the free drug in human melanoma cell line SKMEL-3 (Eskolaky et al. 2015).

2.5.5 Polymeric Nanocarriers

Polymeric nanocarriers include polymeric nanoparticles, dendrimers, polymeric micelles, polymeric drug conjugates, polymersomes, and nanogels.

Polymeric Nanoparticles

Polymeric nanoparticles are solid particles in the range of 10–1000 nm. The drug is encapsulated or dispersed entirely in the polymeric matrix of these nanoparticles. Polymeric nanoparticles can be prepared by different techniques depending on the type of polymer used and the drug to be entrapped. Polymeric nanoparticles are prepared from natural, synthetic polymers with the size range of nanometers (Bennet and Kim 2014). Biomaterials based on natural polysaccharides (dextran, chitosan, alginate, agarose, pullulan) and natural proteins (gelatin, albumin, lecithin, legumin, vicilin) are recently used in nanoparticle preparation. Synthetic polymers such as poly(caprolactone), poly(lactic acid), poly(lactide-co-glycolide) (PLGA), polystyrene, poly(alkyl cyanoacrylate), and poly(methyl cyanoacrylate) are extensively investigated in nanocarrier preparation. Synthetic polymers are more preferred over the natural ones due to their stability and batch-to-batch reproducibility which are major problems that persist with natural polymers (Jawahar and Meyyanathan 2012). Polymeric nanoparticles produce prolonged drug release with

extended circulation time in the blood. The marketed formulations of leuprolide encapsulated in PLGA and camptothecin encapsulated in cyclodextrin-PEG copolymers exhibited controlled release of drugs with decreased toxic effects (Anselmo and Mitragotri 2016; Bobo et al. 2016; Bulbake et al. 2017).

In one study, cyclodextrin-erlotinib complex was loaded into PLGA nanoparticles by multiple emulsion solvent evaporation technique. The complex-loaded PLGA nanoparticles showed threefold higher entrapment efficiency compared to free erlotinib loaded into PLGA nanoparticles. Optimized formulation exhibited approximately 5% drug loading efficiency and sustained-release characteristics. The efficacy studies of cyclodextrin-erlotinib-loaded PLGA nanoparticles in non-small cell lung cancer indicated suppressed colony-forming ability of cancer cells, increased apoptosis, and autophagy inhibition at low IC_{50} values. Additionally, cyclodextrin-erlotinib-loaded PLGA nanoparticles showed superior anticancer activity in the 3D spheroid study compared to plain erlotinib. The study provided significant outcome as erlotinib-resistant lung cancer can be treated with cyclodextrin-modified erlotinib nanoformulations (Vaidya et al. 2019).

A recent study explored the improvement of curcumin therapeutic efficacy in spite of its poor solubility and poor bioavailability by PLGA nanoparticles. Curcumin was loaded into PLGA nanoparticles and its surface was further modified with chitosan and polyethylene glycol. The designed surface-modified curcumin-loaded PLGA nanoparticles showed immensely enhanced bioavailability and prolonged circulation time in the blood. Curcumin-loaded PLGA nanoparticles exhibited superior cytotoxicity and enhanced anti-migratory, anti-invasive, and apoptosis-inducing ability in metastatic pancreatic cancer (Arya et al. 2018).

Dendrimers

Dendrimers are three-dimensional polymer networks that are grown by the successive addition of shells or layers of branched molecules to a central core which is symmetric and nanosized (Abbasi et al. 2014; Shah and Singhvi 2014). Dendrimers are widely synthesized by divergent (center to periphery) and convergent (addition of periphery chains to a central atom or molecule) methods. A variety of polymers including polyamidoamine, arborols, polypropyleneimine (PPI), and polyether-based dendrimers were investigated for cancer therapy (Gupta and Nayak 2015). Dendrimers have been investigated to deliver multiple drugs to tumor tissues. Conjugation of dendrimers with plasma protein or biomolecules could possibly help in prolonging the circulation half-life in the blood and can avoid renal excretion. Cisplatin in conjugation with polyamidoamine dendrimers exhibited high drug loading and increased accumulation of the drug in cancer cells. Additionally, these cisplatin-loaded dendrimers showed lesser systemic side effects compared to free cisplatin. Surface modification has also been investigated for dendrimers. PEGylated lysine dendrimers reported the selective accumulation and enhanced retention of the anticancer agent in the tumor tissues as a result of the enhanced permeation and retention (EPR) effect (Kesharwani and Iyer 2015).

Enzyme-responsive phosphoramidate dendrimers were also studied to improve the localization of dendrimers. These dendrimers are stable in phosphate buffer saline but degrade in the presence of phospholipase C which is overexpressed in cancer cells. This is an attractive strategy to target tumor cells. To minimize the protein binding of these dendrimers in the systemic circulation, the surface can be modified with zwitterionic group such as 2-methacryloyloxyethyl phosphorylcholine which can enhance its blood circulation time. Doxorubicin loaded in phosphoramidate-2-methacryloyloxyethyl phosphorylcholine was designed and investigated for its targeting and drug release. Results showed that the dendrimer formulation was less accumulated in normal cell lines and highly toxic toward cancer cells. In vivo studies in athymic nude mice bearing xenografts of MCF-7 Adriamycin-resistant breast cancer cell line showed improved therapeutic efficacy and reduced toxicity (Zhang et al. 2018) which further confirmed the selectivity of enzyme-responsive phosphoramidate dendrimers.

Polyamidoamine-conjugated chitosan nanoformulation of temozolomide was developed to enhance safe and effective delivery to the brain. The nanoformulation had an average particle size of 201.4 ± 1.70 nm with zeta potential of 24.0 ± 0.17 mV. The in vitro cell line studies on U-251 and T-98G glioma cell lines revealed the improved efficacy of dendrimer formulation compared to free temozolomide. Twofold concentration of temozolomide was accumulated in the brain with dendrimer-based formulation; this was expected due to the modified surface functionality of the formulation which proved the enhanced anticancer efficacy and brain delivery of modified dendrimers (Sharma et al. 2018b).

Polymeric Micelles

Polymeric micelles are the nanocarriers composed of block copolymers which consist of both hydrophobic and hydrophilic blocks. Copolymers may be diblock (poly{lactic acid}-poly{ethylene glycol}) or triblock (poly{ethylene oxide}-poly{propylene oxide}-poly{ethylene oxide}). As these block copolymers are amphiphilic in nature, they self-aggregate into micelles similar to surfactants. Micelle formation mainly depends on the hydrophobic chain bound to the hydrophilic group (Jones and Leroux 1999; Xu et al. 2013). Polymeric micelles are found to be suitable to deliver chemotherapeutic agents to cancerous cells due to their nanosize and surface properties. Polymeric micelles containing anticancer agents are found to exhibit high cytotoxic effects toward cancer cell compared to free drug. Polymeric micelles are also reported for co-delivery of anticancer drugs for effective antitumor activity (Bobo et al. 2016).

To overcome the cisplatin resistance toward cancer cells, poly(ethylene glycol) and polymerized phenylboronic ester-functionalized methacrylate (PBEMA) were used to form block polymer “PEG-b-PBEMA” which can assemble into micelles. The micelles were able to load the hydrophobic drug cisplatin prodrug. In vitro cell line studies revealed 6.1 times higher uptake of platinum micelles in cisplatin-resistant cancer cell line A549R compared to free cisplatin. Intracellular glutathione

plays a key role in cisplatin-resistant cancers. Glutathione concentration was reduced to 32% in case of PEG-b-PBEMA micelles at the phenyl borate-equivalent concentration of 100 μM. The study demonstrated greater efficacy of PEG-b-PBEMA micelles in cisplatin-resistant cancers (Han et al. 2018).

An attempt was made by Emamzadeh and his coworkers for controlled delivery of two drugs, squalene-gemcitabine and paclitaxel. Thermo-responsive block copolymer was synthesized which consists of poly(2-ethylhexyl methacrylate)-b-poly[di(ethylene glycol)methyl ether methacrylate-co-oligo(ethylene glycol)methyl ether methacrylate]. The formed micelles showed drug release in a thermally controlled manner in in vitro cell line studies performed against the pancreatic cell line model (Emamzadeh et al. 2018).

Polymer-Drug Conjugates

Polymeric nanocarriers in which drug molecules are covalently conjugated with water-soluble polymers can act as a prodrug. Polymeric drug conjugates are formed with ester, amide, and disulfide bonds which are stable during drug transportation to the site of delivery (distribution) and easily released at the targeted site by cleavage of the bond at the targeted site. Some polymers for drug conjugates in phase trials for cancer therapy include polyethylene glycol, poly[N-(2-hydroxypropyl) methacrylamide], and poly(glutamate) (Khandare and Minko 2006; Li and Wallace 2008). Polymer-drug conjugates are also investigated for cancer cell targeting. Carboxymethylcellulose conjugated with tetrahydrocurcumin and gemcitabine conjugated with PLGA were studied for targeting colon cancer. Gemcitabine conjugated with PLGA resulted in improved drug stability in the systemic circulation and retained its cytotoxic efficacy toward cancer cells (Plyduang et al. 2014).

In a study, methotrexate was conjugated with polymer poly(glycerol adipate). The drug-polymer conjugate was self-assembled into the nanoparticle, and the size of the nanoparticle was mainly affected by the amount of methotrexate conjugated and pH of the medium. The polymer-drug conjugate was stable in the pH range of 5–9 and ionic strength of 0.15 M sodium chloride. In the pH 7.4 buffer, it was less prone to hydrolysis up to 30 days' duration, but it was enzymatically degradable to release the free drug. Compared to free methotrexate, methotrexate conjugate showed seven times greater efficacy in Saos-2 cells (Suksiriworapong et al. 2018).

An attempt was made by Almawash and his coworkers to inhibit orthotopic pancreatic tumor growth in NSG mice by concomitant administration of docetaxel and cyclopamine by the polymer-drug conjugate. Methoxy-poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol-graft-cyclopamine) and methoxy-poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol-graft-docetaxel) were prepared and self-assembled into micelles together. The size range of the micelles was less than 90 nm, and the com-

bination therapy efficiently inhibited proliferation of MIA PaCa-2 cells and induced apoptosis and cell cycle arrest at M phase compared to monotherapies. In vivo studies in NSG mice revealed improved efficacy in the case of combination delivery compared to monotherapy (Almawash et al. 2018).

Polymersomes

Polymersomes are hollow shell nanoparticles with an aqueous core in the center which is surrounded by a bilayer, prepared from macromolecular diblock, triblock, graft, and dendrimeric copolymers. The bilayer consists of the external and internal hydrophilic environment with a hydrophobic middle environment similar to liposomes. Due to the presence of a hydrophilic core and hydrophobic middle layer, both hydrophilic and hydrophobic drugs (two drugs with different physicochemical properties) can be encapsulated in polymersomes. The membrane of polymersomes is relatively thick and can be modified with some surface-reacting agents. To increase the circulating time, stealth polymersomes have become an attractive approach for multidrug loading in a single carrier (Xu et al. 2005; Meng et al. 2009; Lee and Feijen 2012). In a comparison study of liposomal doxorubicin and polymersome doxorubicin nanocarriers, their dose-related toxicity and side effects were found to be minimum in case of polymersome nanocarrier. However, the therapeutic effects of both nanocarriers were found to be comparable (Du et al. 2012).

Yang and his coworkers developed polymersomes functionalized with selective cell-penetrating peptide for efficient targeted delivery of methotrexate disodium to human lung cancer. The polymersomes were found to be with a small particle size of 63–65 nm. In vitro studies in A549 lung cancer cells showed selective uptake and fast penetration with the efficient release of methotrexate intracellularly. In vivo studies revealed that polymersomes with cell-penetrating peptides showed improved penetration, complete inhibition of tumor progression, and significantly improved survival rates in mice bearing A549 lung tumor xenografts compared to normal polymersome and free methotrexate. This indicated the improved targeting efficiency of modified polymersomes by cell-penetrating peptides (Yang et al. 2018a).

Reduction-responsive chimeric polymersomes were reported for efficient delivery of pemetrexed disodium, a hydrophilic molecule used in lung cancer. CC9 (CSNIDARAC) peptide was used for surface modification of polymersomes to improve specificity toward lung cancer cells. Prepared polymersomes exhibited 22-fold longer circulation time and 9.1-fold higher accumulation in H460 tumor compared to clinical formulation Alimta®. Such stimuli-responsive polymersomes have a wider opportunity in the future to design targeting and safer drug delivery system for cancer treatment (Yang et al. 2018b).

2.5.6 Microemulsion

Microemulsions are stable, clear, isotropic mixtures of oil, water, and surfactant in combination with co-surfactant. Microemulsions are potential carriers which can be administered orally, topically, and parenterally and easy to scale up in a reproducible manner. A wide range of oils is utilized such as oleic acid, castor oil, sesame oil, peanut, eucalyptus oil, and olive oil in the preparation of microemulsion. Various surfactants including polysorbate 20, polysorbate 80, polyoxyl 35 castor oil, polyoxyl 60 castor oil, and PEG 300 caprylic and co-surfactants ethanol, glycerine, poloxamer 407, and propylene glycol have been established for microemulsion preparation and addressed for its stability (Jadhav et al. 2006; Lawrence and Rees 2012).

Microemulsion-based formulations have been investigated for poorly water-soluble and poor bioavailable drugs. In a study, microemulsion of caffeic acid phenethyl ester was formulated and evaluated for cellular uptake and expression of proteins. The microemulsion-based formulation had expressively enhanced the antiproliferative activity against human HCT-116 colorectal and MCF-7 breast cancer cells than that in dimethyl sulfoxide. There was decreased cyclin D1 and increase in p53 which regulate the cell cycle. There was an increase in apoptosis of cancer cells indicating increasing anticancer activity (Chen et al. 2018a).

Chen et al. developed microemulsion of coix seed oil and tripterine and evaluated for cellular uptake and penetration efficiency. Prepared microemulsion surface was modified with transferrin. Prior to surface modification, microemulsion size was found to be 32.47 ± 0.15 nm, and after surface modification with transferrin, particle size was increased to 40.02 ± 0.21 nm. The transferrin-modified emulsion showed 2.58-fold lower IC_{50} than simple microemulsion. Cells treated with transferrin-modified microemulsion had enhanced apoptotic rate up to 1.73 higher compared to microemulsion and 2.77-fold higher with plain tripterine (Chen et al. 2018b).

2.5.7 Solid Lipid Nanocarriers and Nanostructured Lipid Carriers

Solid lipid nanocarriers are alternative to emulsions in which the oil phase is replaced with solid lipid. Solid lipid nanoparticles consist of lipid (triacetin, stearic acid, glyceryl monostearate, triglycerides) and emulsifier (poloxamer, lecithin, polysorbates) (Mukherjee et al. 2009). Nanostructured lipid carriers are advanced to solid lipid nanoparticles to overcome problems like poor drug loading and drug expulsion during storage. Nanostructured lipid carriers contain a lipid mixture of liquid and solid lipid to overcome drug expulsions by irregular lipid arrangements in nanocarriers. Being lipidic in nature, these nanocarriers were found to be more suitable for the delivery of hydrophobic anticancer drugs (Thatipamula et al. 2011; Miller 2013).

Lipidic nanocarriers are reported to improve drug delivery in selective cancer cells. Etoposide-loaded solid lipid nanoparticles for cancer therapy exhibited an increased reduction of tumor cells in both lungs and liver after in vivo administration. Pharmacokinetic studies indicated improved distribution of solid lipid nanoparticle-loaded etoposide for lungs and liver (Athawale et al. 2014). A similar study reported for paclitaxel- and doxorubicin-loaded solid lipid nanoparticles which proved the high cytotoxicity effect of solid lipid nanoparticles toward drug-resistant tumor cells compared to free drug (Miao et al. 2013). The study reported by Taratula et al. showed that nanostructured lipid carriers can be utilized as an effective drug delivery system for cancer. Improved drug delivery to lung cancer cells was observed in the case of nanostructured lipid carriers where normal cells were exposed to the drug to a less extent. These reported studies suggest that solid lipid nanoparticles and nanostructured lipid carriers can be potential nanocarriers for multidrug delivery in cancer therapy (Taratula et al. 2013).

Tamoxifen-loaded solid lipid nanoparticles were prepared and investigated for efficacy against tamoxifen-resistant MCF7 breast cancer cells. In vitro cytotoxic studies demonstrated improved efficacy in resistant cells and altered expression levels of specific miRNA were found. The tamoxifen-loaded solid lipid nanoparticles were found to be more effective compared to free tamoxifen in both MCF7 and MCF7 tamoxifen-resistant cells (Guney Eskiler et al. 2018). Solid lipid nanoparticle-based formulation of sclareol was tested for geno−/cytotoxicity on A549 lung cancer cells. Cytotoxicity study revealed that plain sclareol inhibited A549 with IC_{50} value of 19 μg/ml after 24 h, whereas solid lipid nanoparticle-based formulation persisted even after 48 h (Hamishehkar et al. 2018).

2.5.8 Lyotropic Liquid Crystalline Nanoparticles

Lipid-based liquid crystal nanoparticle systems became an attractive strategy for drug delivery. The amphiphilic lipid molecules are used which self-assemble in an aqueous environment to form three-dimensional nanostructures. These nanoparticulate systems can entrap both hydrophilic and lipophilic drugs and control the drug release from the formulation. Lyotropic liquid crystals are highly ordered, thermodynamically stable, and able to deliver both drug and macromolecule in a single carrier (Singhvi et al. 2018a). Based on preparation method and composition, lipid-based liquid crystal nanoparticles are available as cubosomes, hexasomes, or lamellar type.

Lipid-based liquid crystal nanoparticle has been studied for drug delivery in various therapies. It has been proven as an effective and stable carrier system for cancer cell targeting. Nasr and his coworkers prepared cubosomal nanoparticles of hydrophilic drug 5-fluorouracil for liver targeting. In vitro drug release from cubosomes was high for 1 h followed by controlled release compared to free drug solutions. In vivo biodistribution studies showed that cubosomal formulation had fivefold

increased accumulation of the drug in the liver compared to free drug (Nasr et al. 2015).

Lipid-based liquid crystalline nanoparticle is not only investigated for drug delivery but also for diagnosis and targeting. Multicomponent theranostic lipid-based liquid crystalline nanoparticle has been studied for targeting of different cancers. In a study, folic acid-targeted cubosomes in cancer cell imaging and therapy were evaluated. Normal cubic nanoparticles of etoposide were prepared and its surface was modified with folic acid. Normal cubosomes, folic acid-modified cubosomes, and free drug solution were evaluated for antiproliferative activity. The folate-modified cubosomes exhibited high antiproliferative activity compared to normal cubosomes and free drug solution. The in vitro drug release from cubosomal formulation was sustained for 36 h. In vivo rhodamine B tumor imaging studies demonstrated high tumor targeting efficiency in the case of folic acid-modified cubosomes (Tian et al. 2017).

2.5.9 Nanogel Nanoparticles

Nanogel nanoparticles are a kind of polymeric nanoparticles which can absorb a large amount of water and biological fluids with three-dimensional configurations in submicron size. The presence of hydroxyl amine, hydroxyl sulfate, and carboxyl groups makes them hydrophilic; hence, they are able to absorb a large amount of water (Gonçalves et al. 2010). Polymers including alginate, chitosan, poly(vinyl alcohol), poly(ethylene oxide), poly(ethyleneimine), and poly(vinyl pyrrolidone) are studied for the preparation of nanogel for cancer therapeutics (Hamidi et al. 2008).

2.5.10 Virosomes

Virosomes are non-replicating artificial viruses in which an infectious nucleocapsid is replaced with genes or vaccine antigens required for delivery as they bind more efficiently to infected organ or tissue. Gendicine® for treatment of head and neck squamous cell carcinoma and Rexin-G® for solid tumor treatment are the marketed virosomes (Almeida et al. 1975; Bhaskar et al. 2010).

2.5.11 Metallic Nanoparticles

Inorganic nanoparticles are derived from metals (gold, silver), semiconductors, carbon dots, or iron oxides. Metal nanoparticles may have the potential to deliver the drug at the tumor site and can overcome the problems associated with conventional

chemotherapy. Metallic nanocarriers are stable with a size range of 10–1000 nm. Metal nanoparticles are reported for providing selective tumor cell distribution through passive and active targeting, drug stability, and delivery of small to large molecules for cancer therapy. Additionally, functionalized metal nanoparticles with targeting ligands and theranostic agents offer a novel tool for theranostic application such as imaging, diagnosis, and therapeutic delivery of active agents to tumor-specific cells (Ahmad et al. 2010; Sharma et al. 2018a).

2.5.12 Gold Nanocarriers

Gold nanocarriers are nanoparticulates with a functionalized monolayer of thiol-containing groups around the core gold atoms. These nanocarriers are able to deliver drug molecules or large biomolecules like DNA, proteins, and RNA. The drug release from the gold nanoparticles depends on internal glutathione or pH of the target site or external light stimuli. Gold nanocarriers are mostly studied for targeting drug delivery and diagnostic application as they are safe and monodisperse in size (Hrushikesh et al. 2005; Ghosh et al. 2008).

2.5.13 Carbon Nanotubes

Carbon nanotubes are formed by rolling graphene sheets into a tubular shape and range in size of nanometer to micron meter. Therapeutic agents are conjugated or adsorbed onto nanotubes due to the high surface area of nanocarriers. Carbon nanotubes are insoluble in water, and the tubular structure of these nanocarriers facilitates easy permeation into the target organ. Nanotubes are investigated as a multifunction tool in cancer therapy for drug delivery and tumor diagnosis (Elhissi et al. 2012; Son et al. 2016).

2.6 Targeting Delivery of Nanocarriers

Targeting drug delivery is the localization of the drug to the selective organ, cell, or cell organelle with decreased systemic toxicity. The main importance of nanocarriers is to improve the pharmacokinetic and pharmacodynamic properties of the therapeutic agent. Nanocarriers can be targeted to achieve a desired therapeutic efficacy with minimum toxicity to normal cells. Targeting can be achieved by understanding complete pathophysiology, the microenvironment of tumor cells, and tumor cell biology in detail. Targeting (Fig. 2.4) is broadly classified into three categories: (i) passive targeting, (ii) active targeting, and (iii) physical targeting.

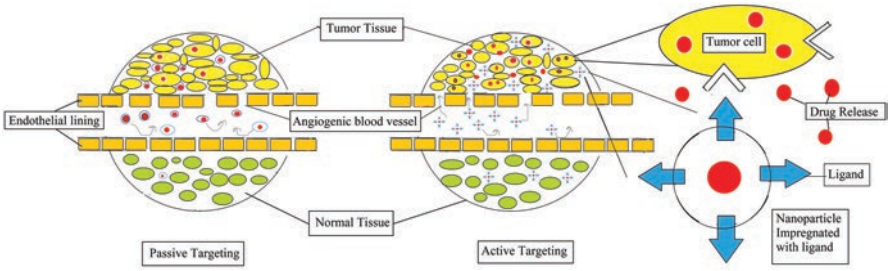

Fig. 2.4 Passive and active targeting approaches for nanocarriers. Large fenestrations and improper formation of the lymphatic drainage system in the tumor environment lead to "enhanced permeation and retention effect." This leads to accumulation of drug moieties in the tumor environment. The tumor microenvironment and biomarkers favor in targeting drug delivery by utilization of active targeting approach. (Figure modified after Danhier et al. 2010)

2.6.1 Passive Targeting

The tumor environment differs from normal cell or tissue conditions which favor the accumulation of drug or carrier in the tumor cell or tissue. It includes "enhanced permeation and retention effect" (EPR) and "reticuloendothelial system uptake" (RES).

Enhanced Permeation and Retention Effect

Fast replicating tumor tissues or cells require high energy and oxygen compared to normal cells which leads to the formation of new blood vessels (angiogenesis). The newly formed blood vessels of tumor cells are irregular with large fenestrations and may lack smooth muscle lining, resulting in leaky vasculature. Moreover, lymphatic vessels are not formed or incompletely formed in the case of tumor cells. The tumor cells have increased the level of vascular endothelial growth factor causing vasodilation which leads to increased blood flow. All these factors lead to increased localization of the drug in the tumor tissue. The leaky vasculature leads to accumulation of nanocarriers and macromolecules in tumor tissue (Fang et al. 2011).

Reticuloendothelial System Uptake

RES organs like the liver and spleen are known for toxic or foreign particle clearance from the body by a well-known mechanism called "opsonization." Due to the lipophilic nature of colloidal systems, they undergo RES uptake and decrease the systemic circulation of colloidal systems. The opsonized particles are accumulated

in RES organs, thereby increasing the drug concentration in tissues. By RES mechanism of the liver or spleen, tumors can be targeted (Danhier et al. 2010).

2.6.2 Active Targeting

Fast-growing tumor tissue differs from normal tissue with respect to the overexpression of receptors and physiological conditions like pH and temperature. As the tumor cells proliferate rapidly, they require high energy which provokes glycolysis, resulting in decreased pH of the tumor environment. pH-responsive nanocarriers are used to target the tumor tissue or cell with varied pH and release of drug – stimulated through a physical change in the polymer used for nanocarrier preparation. Increased angiogenesis in tumor tissue activates endothelial cells with elevated cell adhesion molecules and proteolytic enzymes. Tumor cells overexpress certain biomarkers and proteins which are considered as "tumor-associated antigens" and ligands. These tumor-associated antigens are conjugated with nanocarriers which lead to selective binding to tumor tissue or cell and localization of the drug in the tumor. In many cases, tumor tissue is also overexpressed for folate receptor. Small folic acid molecule has high affinity toward the folate receptor and is used for tumor targeting (Bae and Park 2011). Low-density lipoprotein (LDL) receptors which are meant for cholesterol-rich lipoprotein transport into cells via receptor-mediated endocytosis are overexpressed in tumor tissues. Lipid nanocarriers (liposomes) can be mimicked by lipoproteins for selective tumor cell uptake. Hormone receptors are highly expressed in hormone-dependent tumor tissues like gonadotrophin and luteinizing hormone-releasing organs. Synthetic peptide analogs can be used as targeting agents in tumor tissues which have high affinity to these moieties like breast cancer and prostate cancer. Ligands like monoclonal antibodies and cell-penetrating peptides with an affinity toward tumor tissue can be used as targeting moieties. Nanocarriers attached with ligands get accumulated in the tumor (Dinauer et al. 2005).

2.6.3 Physical Targeting

Ultrasound waves and magnetic field application are the most widely used physical targeting. Polymeric micelle drug delivery systems release the drug at the target site by applying ultrasound, resulting in improved drug accumulation in tumor tissue. Magnetic nanocarriers are used to deliver drugs to the target site by application of an external magnetic field. Magnetic nanoparticles can be more suitable for superficial tumors, whereas in-depth tumor required high applied magnetic field. It is not efficient to accumulate more drug in tumor cells of deeper tissues compared to superficial tissues (Vasir and Labhasetwar 2005).

2.7 Marketed and Nonmarketed Nanocarriers for Cancer Treatment

A number of nanocarrier-based formulations are approved by the Food and Drug Administration as therapeutics and imaging agents. Clinically approved marketed nanoparticles for cancer therapy are summarized in Table 2.2 (Bregoli et al. 2016). The Food and Drug Administration approved current nanoformulations by compiling safety, efficacy, and physicochemical properties through investigational new drug applications. Some of the nanocarriers under clinical studies for cancer therapy are mentioned in Table 2.3 (Anselmo and Mitragotri 2016; Bobo et al. 2016; Bulbake et al. 2017).

In 1995 the Food and Drug Administration approved Doxil®, the first PEGylated liposomal nanocarrier formulation of doxorubicin for cancer therapy (Barenholz 2012). After, other liposomal formulations were approved by the Food and Drug Administration and European Medicine Agencies. Most of the approved formulations listed are of passive targeting formulations. The tumor cell-targeted-based formulations are not yet in the market. However, the passive targeting nanocarriers are found to be at an advantage due to decreased systemic toxicity and accumulation in the tumor environment by enhanced permeation and retention effect. Most of the drugs as nanomedicine in the clinic are encapsulated in liposomes with similar features as approved liposomal formulations. Some of the nanocarriers in clinical trials are approached for targeted drug delivery which indicates the advancement in nanomedicines. ThermoDox® (Celsion Corporation, Lawrenceville, NJ) has recently begun Phase III clinical trials for the treatment of hepatocellular carcinoma. It is a temperature-sensitive liposomal formulation of doxorubicin for targeting liver cancer. The improved number of formulations in clinical trials shows the efforts toward nanocarriers in cancer drug delivery to overcome the system toxicity and improved efficacy of therapeutics (Chang and Yeh 2012; Bregoli et al. 2016).

2.8 Diagnostic Applications of Nanocarriers

Tumor imaging plays a key role in clinical oncology. Many techniques are used conventionally including “computer tomography” and “magnetic resonance imaging,” which focus primarily on deforming and delineating morphological attributes of tumor tissue, such as anatomic location, tumor size, and extent of proliferation using special contrast media (Bhaskar et al. 2010). These techniques, despite being used vastly, face a disadvantage of lower sensitivity. Recent trends have focused on the stimulation of emergence of newer fields of imaging at the molecular level, focusing mainly on diagnosing and imaging various biologically significant events in patients. Recently, fluorescence-mediated tomography, single photon emission tomography, and near-infrared fluorescence-mediated tomography have proven to be versatile for noninvasive tumor imaging. Current advancement in

Table 2.2 Food and Drug Administration-approved nanocarriers for cancer treatment (data compiled from the US Food and Drug Administration website (https://www.accessdata.fda.gov/scripts/cder/daf/)

Brand	Drug	Indication
Liposomes		
DepoCyt®	Cytarabine	Lymphomatous malignant meningitis
Doxil®	Doxorubicin hydrochloride	Acquired immune deficiency syndrome-related Kaposi sarcoma, multiple myeloma, and ovarian cancer
DaunoXome	Daunorubicin	Human immune virus-related Kaposi sarcoma
Marqibo®	Vincristine sulfate	Acute lymphoid leukemia, Philadelphia chromosome-negative, relapsed or progressed
Mepact™	Mifamurtide	Non-metastasizing resectable osteosarcoma
Myocet®	Doxorubicin	Metastatic breast cancer
Onivyde MM-398	Irinotecan	Metastatic pancreatic cancer
PEGylated proteins, polypeptides, aptamers		
Neulasta®	PEGylated filgrastim	Febrile neutropenia, in patients with nonmyeloid malignancies; prophylaxis (subcutaneous)
Oncaspar®	PEGylated L-asparaginase	Acute lymphoblastic leukemia
Polymer-based nanoformulations		
Eligard®	Leuprolide acetate	Advanced prostate cancer
Genexol®	Paclitaxel	Metastatic breast cancer, pancreatic cancer
Opaxio®	Paclitaxel	Glioblastoma
Zinostatinstimalamer®	Styrene-maleic acid conjugated with an antitumor protein NCS	Hepatocellular carcinoma
Protein-drug conjugates		
Abraxane®	Albumin-conjugated paclitaxel	Metastatic breast cancer, non-small cell lung cancer
Kadcyla®	Trastuzumab emtansine	Metastatic breast cancer
Metal-based nanoformulations		
NanoTherm®	Aminosilane-coated superparamagnetic iron oxide 15 nm nanoparticles	Local ablation in glioblastoma, prostate, and pancreatic cancer
Virosomes		
Gendicine®	Recombinant adenovirus expressing wild-type p53	Head and neck squamous cell carcinoma
Rexin-G®	Gene for a dominant-negative mutant form of human cyclin G1 inserted into retroviral core devoid of viral genes	For solid tumors

Table 2.3 Nanocarriers under clinical trials (information compiled from the clinical trial website (Clinicaltrials.gov database)

Name	Drug	Indication
Stimuvax® (phase III)	Tecemotide (BLP25 liposome vaccine)	Breast, prostate, colorectal, and non-small cell lung cancer
Dimericine (phase III)	T4 N5 liposomal lotion,	Skin cancer
ThermoDox® (phase III)	Temperature sensitive Liposomal formulation doxorubicin	Liver cancer
Lipoplatin™ (phase III)	Liposomal formulation of cisplatin	Pancreatic cancer
Aroplatin™ (phase II)	Cis-(trans-R,R-1,2-Diaminocyclohexane) bis (neodecanoate) platinum (II) liposomal formulation	Orphan drug for malignant mesothelioma, metastatic colorectal cancer
Liposomal annamycin (phase II)	Annamycin	Acute myeloid leukemia
SPI-077 (phase II)	Liposomal cisplatin	Lung, head, and neck cancer
OSI-211(phase II)	Liposomal formulation of lurtotecan	Ovarian, head, and neck cancer
S-CKD602 (phase II)	Liposomal belotecan	Advanced small cell lung cancer
LE-SN38 (phase II)	Liposomal SN-38 (active metabolite of irinotecan)	Colorectal cancer
LEP-ETU (phase II)	Liposomal paclitaxel	Ovarian cancer
Endotag-I (phase II)	Liposomal paclitaxel	Breast and pancreatic cancer therapy
Atragen® (phase II)	Liposome composed of tretinoin	Promyelocytic leukemia and other hematologic malignancies
LEM-ETU (phase I)	Liposomal mitoxantrone	Leukemia, breast, stomach, liver, and ovarian cancers
Grab-2	Antisense oligodeoxynucleotide growth factor receptor-based liposomal formulation	Breast cancer and various types of leukemia
INX-0125	Sphingosome encapsulated vinorelbine tartrate	Hodgkin's disease and non-Hodgkin's lymphoma
INX-0076	Sphinogosome-encapsulated Topotecan	Solid tumors
TKM-080301	PLK1 siRNA	Neuroendocrine tumors
Atu027	PKN3 siRNA	Pancreatic cancer
2B3–101	Doxorubicin	Solid tumors
MTL-CEBPA	CEBPA siRNA	Liver cancer
ATI-1123	Docetaxel	Solid tumors
LiPlaCis	Cisplatin	Advanced solid tumors
MCC-465	Doxorubicin	Metastatic stomach cancer
SGT-53	p53 gene	Various solid tumors

(continued)

Table 2.3 (continued)

Name	Drug	Indication
Alocrest	Vinorelbine	Breast and lung cancers
Promitil	Polyethylene glycol liposomal mitomycin C	Solid tumors
NBTXR3 PEP503	External radiation stimulus-based hafnium oxide nanoparticles	For squamous cell carcinoma
Cornell dots	Polyethylene glycol-coated silica nanoparticles	Imaging brain tumors
Magnablate	Iron nanoparticles	Prostate cancer

nanotechnology has certainly shown promising results in noninvasive tumor targeting. With a very small size in a few hundred nanometric range, an increased circulation time is observed since these are not taken up by the liver and kidneys. Research at an extensive scale has certainly proven that nanoparticles do accumulate in the tumor sites because of enhanced permeation and retention effect. These nanoparticles can be fabricated accordingly to obtain the desired size range. These do offer the opportunity to design target-specific smart nanoparticles, multifunctional reagents for imaging, and simultaneous treatment (Zhang et al. 2008).

2.8.1 Quantum Dot Nanoparticles

Nanometric-scale semiconductor quantum dots are light emitting with unique optical and size-tunable optical properties. Quantum dots have improvised signal intensity, brightness, and simultaneous excitation of multiple fluorescence colors. The first feasibility reports of imaging using quantum dots were done for the presence of prostate cancer (Wang et al. 2008). Further, quantum dots emit different wavelengths which collectively can be used for imaging and tracking multiple markers for tumor simultaneously, thus increasing the sensitivity and specificity of the cancer detection.

Quantum dots with near-infrared optical fluorescence technique has recently been developed, where the light penetrates deeper into the tumor tissue and allows a visible comparison in the signal intensity (Chandan et al. 2018). Detection of near-infrared optical fluorescence signals in quantum dots within large animals has been demonstrated. However, cadmium is the prime component of this which has certainly raised the issue of potential toxicity, leading to questionable clinical future (Johannsen et al. 2007).

2.8.2 Nanoparticles of Magnetic Iron Oxide

These are gaining an increasing attractiveness as the potential precursors for magnetic resonance imaging contrast agents. These possess unique properties of paramagnetic nature. Iron oxide nanoparticles have shown prolonged retention time and generally have a biodegradable nature and are considered to be less toxic (Weinstein et al. 2010). Many of these iron oxide nanoparticles are proven to be safe in human clinical trials. Studies have also reported that iron oxide nanoparticles may be internalized and allow the magnetic labeling of targeted cells. The toxicity concern for these iron oxide nanoparticles is very low; thus, extensive research is being carried out on these. Recent advancements focus on the development of magnetic nanoprobes of ultrasensitive tumor imaging. This newer generation of nanoparticles of iron oxide should leave us with powerful potential in contrast and imaging (Laurent et al. 2008).

2.9 Regulatory Considerations

Nanocarriers alter the pharmacokinetic and pharmacodynamic properties of the drug molecule. Due to the nano-range size, solubility and permeability of the molecule can be enhanced. Improved efficacy of molecules by nanocarriers brought exciting developments in a short time. The US Food and Drug Administration and European Medicines Agency have made several amendments in the drafted guidelines from the date of issue. The inadequate guidelines on nanocarrier characterization, biodistribution, shelf-life, and impurity establishment are the major limiting step apart from the regulatory guidelines. Scale-up, reproducibility, and optimum particle size distribution achievement are difficult in the manufacturing of nanoparticles. To overcome these hindrances, a separate drafted guideline is allotted for liposomes, polymeric micelles, nano-iron, and surface-coated nanoparticles by the European Medicines Agency and Food and Drug Administration although submissions are indistinguishable (Subin et al. 2018).

2.10 Future Perspective

Nanocarrier-based drug delivery systems have potential with a wide range of applications in cancer therapy. They can overcome multidrug resistance and demonstrate improved efficacy of the drug by targeting ability with reduced cytotoxic effects. Despite the numerous advantages, there is a need to come across the probable chronic and acute toxic effects of nanocarriers in tumor therapy (Huang et al.

2017). For the past two decades, extensive research has led to the filing of more than 1500 patents and some have completed clinical trials. The consistent uniformity of size, drug encapsulation, and drug loading are to be explored to a large extent. The clarification in the molecular level of the disease may lead to advances in nanocarrier application (Patra et al. 2018). Novel technologies like microneedle-based systems can be utilized for site-specific delivery of nanocarriers in skin and breast cancer. This can improve the local delivery of drugs with reduced side effects and the stability of nanocarriers and overcome formulation-related issues like aggregation (Waghule et al. 2019). Conjugation of new technologies like 3D printing with nanotechnology may lead to innovative nanomedicine. The nanocarriers produced by these advanced techniques may be useful in tailoring drug release and dose of the drug and improve chemical stability (Beck et al. 2017; Singhvi et al. 2018c).

2.11 Conclusions

Cancer still retains its position as the leading cause of morbidity worldwide. However, an improvement in patient health and survival is seen as a result of increased research efforts over the past 20 years. The timeline for cancer treatment has seen deviations ranging from simple non-discriminating cytotoxic molecules to specific targeted efficient formulations. The identification of targets of newer origin with a specific therapeutic indication has resulted in the development of tumor target-based medications. The current chemotherapy aims to increase outcomes for cancer patients. Achieving this goal is dependent on the capability to target and destroy the cancerous cells and affecting the least the surrounding healthy cells. Nanotechnology-based drug delivery approaches have been tried out by various scientists to improvise the conventional regimen of chemotherapy. Also, in this chapter various nanocarrier-based drug delivery strategies have been described to overcome the physicochemical and physiological origin. These must be surpassed in order to deliver medications to tumor cells. Nanoformulation approach has also led to improvisation in safety concern of some drugs, so that higher doses may be incorporated.

The researchers are quite near in revealing the genetic makeup for many common types of cancer. With the increase in the current status of scientific knowledge, treatments are basically modified accordingly. The collaborative evolution of various disciplines including biology, medicines, physics, chemistry, and engineering technology with nanomedicine has certainly led to stability in the concrete pavement of these “magic bullets.”

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Chapter 3
Gold Nanoparticle-Mediated Delivery of Therapeutic Enzymes for Biomedical Applications

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Contents

Abstract Nanobiotechnology application, at the interface of nanocarrier and therapeutic enzyme, holds great promises in the nanomedicine. In this direction, gold nanocarriers contribute a plethora of nanobiotechnological applications due to their unique properties. The salient features of gold nanoparticle include high catalytic activity, unique optical properties, ease of surface functionalization, biocompatibility and long-period stability. The potential use of gold nanoparticle in conjunction with therapeutic enzymes can be further extended for curing many dreadful diseases.

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We reviewed the suitability of gold nanocarrier-bound therapeutic enzyme delivery in biomedical modality, in particular to therapeutic application. The major health issues such as cancer, cardiovascular disease and brain disease are regulated with the intervention of gold nanoparticle-bound therapeutic enzyme delivery. Gold nanocarrier-bound therapeutic enzyme has increased the pharmacokinetic and pharmacodynamic correlation in drug delivery. Therapeutic fungal asparaginase covalently immobilized on the surface of gold nanoparticles demonstrated higher cytotoxicity effect against lung cancer and ovarian cell lines. It is further demonstrated that the gold nanoparticle-bound asparaginase has increased its bioavailability up to 85% more against lung cancer. The serratiopeptidase-bound gold nanoparticle has considerably increased anti-inflammatory response. The present chapter is concluded with recent literature discussion that gold nanoparticle-bound therapeutic enzyme has broadened the scope of traditional therapeutics to effective therapeutic enzyme delivery.

Keywords Nanogold · Biogenic methods · Therapeutic enzyme · Enzyme as a drug · Bioconjugation · Stability · Applications · Cell lines · Drug delivery · Anti-inflammatory · Cancer

3.1 Introduction

Nanomaterials, in particular gold nanoparticles, have gained attention due to the simplicity in its mode of action, ease of surface modifications, a plethora of applications such as data storage, environment, especially in medical biotechnology as nanocarrier for enzyme immobilizations and for drug delivery (Chamundeeswari et al. 2018; Golchin et al. 2018; Kaphle et al. 2018; Dykman and Khlebtsov. 2017; Verma 2017a, b, c, d; Gupta et al. 2016; Shankar et al. 2015; Kumar et al. 2014a, b; Sharma et al. 2014a, b; Verma et al. 2013a, b, c, d). Drug delivery is a fascinating field of scientific research in nanobiotechnology. Drug delivery is defined as the process for the release of biologically active medicament at a definite speed and at a destined location (Xin et al. 2017). Functionalized gold nanocarriers present huge probabilities for multiple, locus-specific drug delivery to the disease locus as their diminutive size can effectively penetrate across obstacles through small capillaries into individual cells. Specifically, gold nanoparticles have revealed great capacity to be used as drug delivery platforms (Pelaz et al. 2017). Gold nanoparticles have tremendous potential to deliver multiple drug molecules, recombinant proteins, vaccines and nucleotides into their targets effectively. Targeted/localized drug delivery is possibly achieved through active or passive approaches. Active targeting is based on conjugating the therapeutic agent or carrier system to a cell- or tissue-specific ligand, whereas passive targeting is based on a therapeutic agent that passively reaches out to a localized organ for efficient biomedical application such as target tumours by incorporation in the macromolecule or nanoparticle (Daraee et al. 2016).

Gold nanoparticles have always been considered as potential target for localized drug delivery applications in the field of biomedicine (Baskar et al. 2018). Nanocarriers have unique physicochemical characteristics such as definite size, surface area to mass ratio, chemical stability with high reactivity and functionalized structure with admirable biocompatibilities (Kong et al. 2017). Today, nanocarriers can serve as drug depots exhibiting prolonged-release kinetics and long persistence at the target site. Nanotechnology-based biomedicines have improved the pharmacokinetic and pharmacodynamic potential of different drug molecules which are capable of targeted/localized drug delivery applications such as early detection of cancer lesions, determination of molecular signatures of the tumour by non-invasive imaging and, most importantly, molecular-targeted cancer therapy and cardiovascular and neurodegenerative disease treatments (Pietro et al. 2017). Biocompatibility of gold nanoparticles, with ease of their biological and chemical nature, mimics the function of some enzymes including superoxide dismutase, esterase, peroxidase and glucose oxidase for various therapeutic applications such as tissue regeneration (Golchin et al. 2018). Localized delivery of drug-coated nanoparticles and emergence of such nanotherapeutics/diagnostics based on therapeutic enzymes provides the way for deeper understanding of human longevity and human ills that include genetic disorders, cancer and cardiovascular disease (Peer et al. 2007).

The present article is focussed on the applications of gold nanoparticle-mediated therapeutic enzyme delivery. Various physicochemical and biological methods of gold nanoparticle synthesis, biotechnology of therapeutic enzyme production, strategies of robust nanocarrier-enzyme bioconjugate development and biomedical applications of the gold nanocarrier-bound therapeutic enzyme are critically discussed.

3.2 Synthesis of Gold Nanoparticles

Various methods such as the Turkevich method, Brust-Schiffrin method, seeding growth method and biological method have been employed for the synthesis of gold nanoparticles (Herizchi et al. 2016; Rawat et al. 2016; Abdulghani and Hussain 2014; Singh et al. 2013; Siti et al. 2013; Bisker et al. 2012; Chithrani et al. 2010; Akbarzadeh et al. 2009; Mohanpuria et al. 2008; Brust et al. 1994; Turkevich et al. 1951).

Various chemical and physical methods of gold nanoparticle synthesis are most commonly used. However, these chemical methods involve the use of expensive and hazardous chemicals under extreme reaction conditions (Ahmed et al. 2015a; Ahmed et al. 2015b; Krishnaswamy et al. 2014; Kumar et al. 2011a, b). In addition, these nanoparticles may have harmful effects in biomedical applications (Noruzi et al. 2011; Shankar et al. 2004a, b). To overcome these problems, green synthesis of nanoparticles is an emerging field of research in the current era (Kulkarni and Muddapur 2014; Mittal et al. 2013). Hence, there is a growing need to develop eco-friendly and cost-effective procedures for the synthesis of nanoparticles. The inherent,

clean, nontoxic and environment-friendly ability of microorganisms and plant systems to synthesize the gold nanoparticles is particularly important in the advancement of nanobiotechnology (Mohanpuria et al. 2008).

Recently, plants are commonly employed for the synthesis of gold nanoparticle (Table 3.1A). The biosynthesis of gold nanoparticles using plants and plant extracts is a very important aspect due to lack of pathogenicity and their diversity (Chandran et al. 2014). Green synthesis of nanoparticles uses extracts of various plants such as *Aloe vera* (Chandran et al. 2006), *Pogostemon benghalensis* (Paul et al. 2015), *Salix alba* (Ul et al. 2015), *Solanum nigrum* (Muthuvel et al. 2014), *Terminalia arjuna*

Table 3.1A List of different plants employed for the synthesis of gold nanoparticles

Name of plants	Size of gold nanoparticles	References
Gymnocladus assamicus	4–22 nm	Tamuly et al. (2013a, b)
Cacumen platycladi	Variable	Wu et al. (2013)
Pogostemon benghalensis	13 nm	Paul et al. (2015)
Mangifera indica	6–18 nm	Yang et al. (2014)
Coriandrum sativum	6–57 nm	Narayanan and Sakthivel (2008)
Nerium oleander	2–10 nm	Tahir et al. (2015)
Butea monosperma	10–100 nm	Patra et al. (2015)
Arachis hypogaea	110–130 nm	Raju et al. (2014)
Solanum nigrum	50 nm	Muthuvel et al. (2014)
Hibiscus cannabinus	10–13 nm	Bindhu et al. (2014)
Sesbania grandiflora	7–34 nm	Das and Velusamy (2014)
Salix alba	50–80 nm	Ul et al. (2015)
Eucommia ulmoides	NA	Guo et al. (2015)
Galaxaura elongata	3–77 nm	Abdel-Raouf et al. (2017)
Ocimum sanctum	30 nm	Philip et al. (2011)
Torreya nucifera	10–125 nm	Kalpana et al. (2014)
Olea europaea	50–100 nm	Khalil et al. (2012)
Rosa indica	23–60 nm	Manikandan et al. (2014)
Pistacia integerrima	20–200 nm	Islam et al. (2015)
Terminalia arjuna	60 nm	MohanKumar et al. (2013)
Euphorbia hirta	6–71 nm	Annamalai et al. (2013)
Morinda citrifolia	12–38 nm	Suman et al. (2014)
Ziziphus mauritiana	20–40 nm	Sadeghi (2015)
Aloe vera	2–8 nm	Chandran et al. (2006)
Cassia auriculata	15–25 nm	Kumar et al. (2011a, b)
Hibiscus rosa-sinensis	16–30 nm	Philip (2010)
Ananas comosus	10–11 nm	Bindhu et al. (2014)
Sapindus mukorossi	9–19 nm	Reddy et al. (2013)
Prunus domestica	14–26 nm	Dauthal and Mukhopadhyay (2012)
Magnolia kobus	5–300 nm	Song et al. (2009)
Coleus amboinicus lour	9–31 nm	Narayanan and Sakthivel (2010)
Gnidia glauca	50–150 nm	Ghosh et al. (2012)

NA: not available

(MohanKumar et al. 2013), *Piper pedicellatum* (Sujitha and Kannan 2013), *Terminalia chebula* (Tamuly et al. 2013a, b), *Citrus reticulata* and *Citrus sinensis* (Mittal et al. 2013), *Mangifera indica* (Philip et al. 2011), *Murraya koenigii* (Das et al. 2011), *Zingiber officinale* (Kumar et al. 2011a, b), *Cymbopogon citratus* (Parida et al. 2011; Smithaa et al. 2009), *Coriandrum sativum* (Narayanan and Sakthivel 2008), *Azadirachta indica* (Shankar et al. 2004a, b) and *Medicago sativa* (Gardea-Torresdey et al. 2002). Plant extracts may act as both reducing agent and stabilizing agent in the synthesis of nanoparticles. In view of its simplicity, the use of plant extract for reducing metal salts to nanoparticles has attracted considerable attention (Mittal et al. 2013). Large-scale biosynthesis of nanoparticles is a main factor in green syntheses in which suitability of the reagents plays an important role (Chandran et al. 2014). Gold nanoparticles are rapidly synthesized using aqueous leaf extracts of *Acalypha indica* and *Azadirachta indica* as novel sources of bio-reductants (Krishnaraj et al. 2014). Biosynthesis of gold nanoparticles using leaf extracts of *Zingiber officinale,* which acted as a reducing and capping agent, was also reported (Singh et al. 2011). The use of plants and plant extracts for the preparation of gold nanoparticles is more advantageous. It does not require elaborate processes such as intracellular synthesis and multiple purification steps.

The biological method for the synthesis of nanoparticles by using microbes like bacteria, fungi, actinomycetes, yeast and algae is providing a wide range of resources for the synthesis of nanoparticles (Table 3.1B). Use of diverse microorganisms such as *Bacillus marisflavi* (Nilofar and Shivangi 2016), *Bacillus subtillus* (Reddy et al. 2010),

Table 3.1B List of different microorganisms employed for the synthesis of gold nanoparticles with different sizes

Type	Name	Size	References
Bacteria	*Bacillus subtilis*	5–25 nm	Reddy et al. (2010)
	Pseudomonas aeruginosa	5–30 nm	Husseiny et al. (2007)
	Escherichia coli	25–33 nm	Du et al. (2007)
	Rhodopseudomonas capsulata	10–20 nm	Shiying et al. (2007)
	Stenotrophomonas maltophilia	40 nm	Nangia et al. (2009)
	Brevibacterium casei	10–50 nm	Kalishwaralal et al. (2010)
	Bacillus licheniformis	10–100 nm	Kalishwaralal et al. (2009)
	Pseudomonas veronii	5–25 nm	Baker and Satish (2015)
	Klebsiella pneumoniae	35–65 nm	Malarkodi et al. (2013)
	Marinobacter pelagius	20 nm	Sharma et al. (2012)
	Geobacillus sp.	5–50 nm	Correa-Llantén et al. (2013)
	Bacillus marisflavi	14 nm	Nilofar and Shivangi (2016)
Fungi	*Rhizopus oryzae*	9–10 nm	Mukherjee et al. (2002)
	Fusarium oxysporum	8–40 nm	Das et al. (2012)
Algae	*Shewanella algae*	10 nm	Ogi et al. (2010)
	Sargassum wightii	8–12 nm	Singaravelu et al. (2007)
	Chlorella vulgaris	NA	Xie et al. (2007)
	Galaxaura elongate	3–77 nm	Abdel-Raouf et al. (2017)

NA: not available

Bacillus licheniformis (Kalishwaralal et al. 2009), *Pseudomonas veronii* (Baker and Satish 2015), *Galaxaura elongata* (Abdel-Raouf et al. 2017), *Chlorella vulgaris (*Xie et al. 2007), *Trichoderma asperellum* and *Trichoderma reesei* (Vahabi et al. 2011; Mukherjee et al. 2008), *Fusarium oxysporum* (Das et al. 2012), endophytic fungus *Verticillium* sp. (Bharde et al. 2006) and *Rhizopus oryzae* (Mukherjee et al. 2002) was employed for the synthesis of gold nanoparticles. It is a relatively new area of research with considerable prospects that can be used either extracellularly or intracellularly due to their innate potential. Mukherjee et al. (2002) also demonstrated that fungi secrete a significantly higher amount of proteins than bacteria; this would amplify the productivity of nanoparticle synthesis. Further, it is environmentally acceptable, economic, time saving and easily scaled up. Due to this ability to adapt to extreme conditions, these fungi can be used as a potential resource for biosynthesis of nanoparticles.

It can be inferred from the above-stated various methods of gold nanoparticle synthesis that the biological route provides an attractive possibility for the scale-up of gold nanoparticle production.

3.3 Biotechnology of Therapeutic Microbial Enzymes

Enzymes are the excellent biocatalysts that catalyse complex chemical reactions under appropriate physiological conditions. Enzymes possess a unique chiral-selective property, a prerequisite step for enantiomerically pure pharmaceutical drug production (Mane and Tale 2015; Bankar et al. 2009; Underkofler et al. 1957). Use of enzymes as drug target exhibits advantages over conventional drugs due to their unique target specificity and multiple substrate conversion (SKumar and Abdulhameed 2017). Therapeutic enzymes are obtained from bacteria, fungi and yeast (Table 3.2). Microbial enzyme production offers cost-effective technology that has a potential profitable market (Mane and Tale 2015; Gurung et al. 2013; Teal and Wymer 1991). Nowadays therapeutic enzymes are used for treating a diverse spectrum of life-threatening diseases such as cancer and gastrointestinal disorders and enzyme replacement therapy. Thus, therapeutic enzymes served as oncolytics, thrombolytics or anticoagulants and anti-inflammatory agents (Mane and Tale 2015; Gurung 2013; Gurung et al. 2013; Vellard 2003; Ozcan et al. 2002; Gonzalez and Isaacs 1999).

3.3.1 Different Types of Therapeutic Enzymes

Specificity of therapeutic enzymes makes them the most desirable therapeutic agents for the treatment of various diseases. Digestive and metabolic enzymes can be used either alone or in combination with other therapies for treating a variety of

Table 3.2 List of therapeutically important microbial enzymes employed for drug delivery

Microbial enzyme/source	Applications	References
Nattokinase/*Bacillus subtilis*	Cardiovascular disorder treatment	Dabbagh et al. (2014)and Hsia et al. (2009)
Uricase/Aspergillus flavus	Gout treatment	Terkeltaub (2009)
Superoxide dismutase/*Mycobacterium* sp., *Nocardia* sp.	Anti-inflammatory action	Ethiraj and Gopinath (2017) and Kaur and Sekhon (2012)
Serratiopeptidase/*Serratia marcescens*		
Glucosidase/*Aspergillus niger*	Cancer treatment	Ahmed et al. (2017), Dubey et al. (2015), Sharma et al. (2014), Yu et al. (2013), Kaur and Sekhon (2012), Jain et al. (2012), Para et al. (1984), Spiers and Wade (1976)and Peterson and Ciegler (1969)
L-Methionase/*Pseudomonas* sp.		
Arginase/*Bacillus subtilis*, *E. coli*		
Asparaginase/*E. coli*		
Glutaminase/*E. coli*, *Bacillus subtilis*		
Tyrosinase/*Streptomyces glaucescens, Erwinia herbicola*		
Staphylokinase/*Staphylococcus aureus, Streptococci* sp.	Anticoagulant action	Vakili et al. (2017), Kaur and Sekhon (2012), Zaitsev et al. (2010) and Banerjee et al. (2004)
Streptokinase/*Streptococci* sp.		
Urokinase/*Bacillus subtilis*		

diseases safely (Mane and Tale 2015; Kaur and Sekhon 2012; Sabu 2003; Vellard 2003; Cooney and Rosenbluth 1975).

Demands of therapeutic enzymes are growing rapidly due to massive biomedical applications. At present, the most prominent medical uses of microbial enzymes are the removal of dead skin and burns by proteolytic enzymes and clot busting by fibrinolytic enzymes (Singh et al. 2016). For example, a good agent for thrombosis therapy is nattokinase, a potent fibrinolytic enzyme (Sumi et al. 1987). Enzymes, namely, L-asparaginase, L-glutaminase, L-tyrosinase and galactosidase, are used as antitumour agents, and streptokinase and urokinase act as anticoagulants. Acid protease, dextranase and rhodanase may be used to treat alimentary dyspepsia, tooth decay and cyanide poisoning, respectively (Okafor, 2007). Microbial lipases and polyphenol oxidases are involved in the synthesis of diltiazem intermediate (2R,3S)-3-(4-methoxyphenyl)methyl glycidate and 3,4-dihydroxylphenyl alanine (DOPA, for treatment of Parkinson's disease), respectively (Faber 1997). Tyrosinase, an important oxidase enzyme, is involved in melanogenesis and in the production of L-DOPA. Dopamine, a potent drug to control the myocardium neurogenic injury and for the treatment of Parkinson's disease, is produced using L-DOPA as a precursor (Zaidi et al. 2014; Ikram-ul-Haq and Qadeer 2002). Chitosanase catalyses hydrolysis of chitosan to biologically active chitosan oligosaccharides, which are used as antimicrobial and antioxidant, in lowering blood cholesterol and high blood pressure, controlling arthritis, protecting against infections and improving antitumour

properties (Thadathil and Velappan 2014; Zhang et al. 2012; Ming et al. 2006; Kim and Rajapakse 2005).

3.3.2 Therapeutic Enzyme Production

In the pharmaceutical industry, bioprocessing of enzymes for use as drugs is an important aspect that is now being capitalized at every research and development centre across the globe (Cassileth 1998). Microbial therapeutic enzymes offer economic feasibility. That is why the use of microbial enzymes is increasing day by day (Gurung et al. 2013). Various methods involving fermentation technology are available for the production of microbial enzymes (Sabu et al. 2000). These include solid-state fermentation and submerged fermentation. On commercial scale, these methods are utilized for mass production of therapeutic enzymes than liquid cultures in huge bioreactors (Lozano et al. 2012). These important enzymes can be produced by different methods of fermentation. On an industrial scale, liquid cultures in huge bioreactors are preferred for producing therapeutic enzymes in bulk. Other processes like solid-state fermentations and submerged fermentations are also widely used for the production of therapeutic enzymes (Sabu 2003). Large-scale productions of microbial therapeutic enzymes using various production techniques and downstream processing have been reported (Sabu et al. 2005; Sabu 2003).

Gold nanoparticle was employed for some enzyme deliveries such as superoxide dismutase, esterase, peroxidase and glucose oxidase for various therapeutic applications (Golchin et al. 2018). It is very pertinent that only a few therapeutic enzymes have been explored for gold nanoparticle-mediated drug delivery so far. Thus, it can be inferred that many therapeutic enzymes have to be employed for nanocarrier-mediated drug delivery.

3.4 Methods for Developing Robust Gold Nanocarrier for Therapeutic Enzyme Delivery

Therapeutic enzymes are susceptible to denaturation under harsh environmental conditions (Abraham et al. 2014; Puri et al. 2013; Verma and Kanwar 2010, 2012; Verma et al. 2009, 2011, 2012). In order to make a robust and biocatalytic stable enzyme, enzymes need protection and cost-effective recyclability by immobilizing the suitable inert carrier (Verma et al. 2016). Nanomaterials possess many physicochemical advantages over their bulk materials. Immobilization of enzymes on the nanoparticles holds a great promise to improve their functionality and biocatalytic potentials. Nano-immobilization methods are generally categorized into four types, namely, (1) electrostatic adsorption, (2) conjugation of the ligand on the nanoparticle surface, (3) conjugation to a small cofactor molecule that the protein can recognize and bind to and (4) direct conjugation to the gold nanoparticle surface (Fig. 3.1;

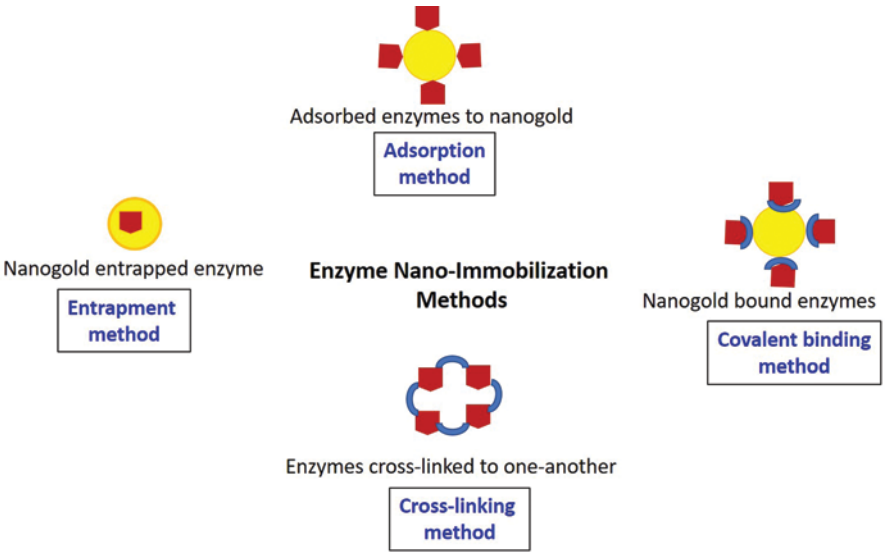

Fig. 3.1 Schematic of four methods of enzyme nano-immobilization with gold nanoparticle

Table 3.3 Pros and cons of the enzyme nano-immobilization methods

Type of immobilization method	Advantages	Disadvantages	References
Adsorption method	Simple and chemical free method, no confirmation of the enzyme	Weak bonding may cause enzyme leakage (desorption) from the nanocarrier	Verma et al. (2016), Kanwar and Verma (2010), Kanwar et al. (2007a, b)
Entrapment method	Enzyme protection, ease of separation	Possibility of enzyme leakage, low enzyme loading	Kadri et al. (2018) and Verma et al. (2016)
Cross-linking method	High enzyme loading, strong binding	Possibility of alteration in enzyme active site, loss of enzyme activity	Velasco-Lozano et al. (2016) and Verma and Barrow (2015)
Covalent binding method	Strong enzyme binding, leakage-free enzyme binding	Chemical modification of enzymes, enzyme denaturation	Kumar et al. (2014a, b), Abraham et al. (2014) and Verma et al. (2013a, b, c, d)

Verma et al. 2016; Verma and Barrow 2015; Puri et al. 2013; Yeh et al. 2012; Ackerson et al. 2010; Aubin and Hamad 2008). Each of these techniques has its pros and cons (Table 3.3; Kanwar and Verma 2010; Kanwar et al. 2008; Kanwar et al. 2007a,b; Kanwar et al. 2006; Kanwar et al. 2005). Thus, sometimes a combination of these nano-immobilization techniques is employed in order to get robust gold nanoparticle conjugates.

Among different nanoparticles (fullerenes, single-walled and multiwalled carbon nanotubes, magnetic nanoparticles, modified silicon nanowires, dendrimers and quantum dots), only gold nanoparticles can be marked as the most used and widespread for biomedical applications (Zhang et al. 2015). Besides the common properties typical for nanomaterials, the main specific characteristics of gold nanostructures are its stability for a long period of time, easy surface functionalization, biocompatibility, unique optical properties and high catalytic activity providing the successful use of gold nanoparticles (Yu et al. 2016). Gold nanoparticles can be attached to those functional groups which have positive charge because of negative charge on their surface. Likewise, the presence of six free electrons in the conduction band of gold nanoparticles makes them potential candidates to bind with reactive functional groups like thiols and amines. Silica, aluminium oxide and titanium oxides facilitate the attachment of different functional groups on the surface of gold nanoparticles (Sharma et al. 2015; Sharma et al. 2010; Kim et al. 2010; Sun et al. 2008; Tkachenko et al. 2004). Thus, gold nanoparticles can be easily tagged with various proteins and biomolecules that are rich in amino acids (Giljohann et al. 2010; Eustis and El-Sayed 2005). Therapeutic and diagnostic efficiency can strongly be influenced by changing the surface characteristics of nanoparticles such as size, shape and surface charge which in turn change cellular uptake and functional surface area (Jazayeri et al. 2016).

The conjugation of different functionalized groups to nanoparticles is prerequisite for improving stability, functionality and biocompatibility (Delong et al. 2010). It has also been reported that it is possible to control the interactions of gold nanoparticles with cell membranes in order to improve their cellular uptake while minimizing their toxicity by rigid change of the surface charge densities (Lin et al. 2010). Physical and chemical interactions are used for attaching functional groups (DNA, RNA, enzymes, peptides, bovine serum albumin, polyethylene glycol and proteins) to gold nanoparticles' surface (Cho et al. 2012; Lee et al. 2008). Noncovalent interaction between functional groups and gold nanoparticles depends on three phenomena: (a) ionic attraction between the negatively charged gold and the positively charged functional group, (b) hydrophobic attraction between the functional group and the gold surface and (c) dative binding between the gold conducting electrons and functional group. Covalent interactions between functional groups and nanoparticle surface are achieved in a number of ways like (i) through chemisorption via thiol derivatives, (ii) through the use of bifunctional linkers and (iii) through the use of adapter molecules like streptavidin and biotin (Delong et al. 2010). Other functional groups like citrate, tannic acid and polyvinylpyrrolidone can be capped to gold nanoparticles (Marcelo et al. 2015; Senoudi et al. 2014; Mirza and Shamshad 2011).

Gold nanoparticles are useful for important biomedical applications including targeted drug delivery, cellular imaging and biosensing (Hwang et al. 2012; Hong et al. 2012; Giljohann et al. 2010; Huang and El-Sayed 2010). In a recent study, therapeutic fungal asparaginase was covalently immobilized on the surface of gold nanoparticles or nanoporous gold nanoparticles (Baskar et al. 2018). Immobilized gold nanoparticle was further targeted for drug delivery with respect to cancer treat-

ment. It has been demonstrated that the synthesized gold nanobiocomposite of asparaginase can be used as an effective anticancer drug with increased bioavailability against lung cancer.

Gold nanoparticles proved robust nanocarriers for neurotrophin peptides (Patrizia et al. 2017). The immobilization of neurotrophin peptide was achieved by direct physisorption and lipid bilayer-mediated adsorption methods. The nanobioconjugates were characterized by UV-vis spectroscopy, X-ray photoelectron spectroscopy, dynamic light scattering, zeta-potential analyses and atomic force microscopy. Both peptide- and lipid-dependent features were identified to have a modulation in the peptide coverage of nanoparticles as well as in the cellular uptake of nerve growth factors and brain-derived neurotrophic factors. Robust hybrid gold peptide nanointerface demonstrated a promising approach to neurotrophin for crossing blood-brain barriers. Gold nanocarrier provided **new multipotential therapeutic nanoplatform for the treatment of** central nervous system **disorders.**

Gupta et al. (2016) reported a new generation of surface ligands based on a combination of short oligo(ethylene glycol) chains and zwitterions capable of providing non-fouling characteristics while maintaining colloidal stability and functionalization capabilities. Moreover, conjugation of gold nanoparticles with avidin helped in the development of a universal toolkit for further functionalization of nanomaterials.

Muthurasu and Ganesh (2016) prepared glucose oxidase-stabilized gold nanoparticles by changing the pH and showed feasibility of employing such nanocarrier as an ideal sensor for dual-mode sensing of glucose. Gold nanoparticles were able to detect glucose at a low concentration with high sensitivity, good stability and reproducibility suggesting promising applications in the field of nanobiosensors.

Malda et al. (2010) developed a conjugate of gold nanoparticle and therapeutically important superoxide dismutase at specific physiochemical reaction condition. Binding of enzyme-nanoparticle was confirmed by gel electrophoreses. Superoxide dismutase is a metalloenzyme that catalysed the dismutation of superoxide radicals into hydrogen peroxide and oxygen. Reactive oxygen species, such as superoxide radicals, are the root cause to pathogenesis of several diseases, such as familial amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, Down syndrome and several neurological disorders (Halliwell and Gutteridge 2012; Pissuwan et al. 2007). Gold nanoparticle-superoxide dismutase enzyme conjugates proved its therapeutic potential in the prevention of oxidative damage from superoxide radicals (He et al. 2013; Zhao et al. 2012).

Synthesis of gold nanoparticles using the therapeutic enzyme serratiopeptidase was done at 25 °C and physiological pH 7 (Venkatpurwar and Pokharkar 2010). The formation of serratiopeptidase-reduced gold nanoparticles was confirmed by UV-visible spectroscopy, transmission electron microscopy, X-ray diffraction and Fourier transform infrared spectroscopy. This study successfully demonstrated that physiological condition is an important process parameter for the controlled synthesis of highly stable gold nanoparticles with respect to retention of biocatalyst activity. Researchers further confirmed use of gold nanoparticle as a carrier for

serratiopeptidase led to an improved anti-inflammatory response (Venkatpurwar and Pokharkar 2010).

It is inferred from the above-stated studies that the binding of gold nanocarrier either non-covalently or covalently to therapeutically important enzyme depends on the immobilization reaction conditions and enzyme stability. This is a very critical step to immobilize fragile enzyme on the non-functionalized surface of gold nanoparticle. Robust gold nanocarrier immobilized enzyme successfully demonstrated various biomedical applications such as neurological and inflammatory issues.

3.5 Potential Applications of Gold Nanocarriers in Enzyme-Mediated Drug Delivery

Gold nanoparticle-based targeted drug deliveries have considerable applications to overcome the limitations in traditional therapeutics (Daraee et al. 2016). For example, antineoplastics, antiviral drugs and various other types of drugs are manifestly stuck due to their inability to cross the blood-brain barrier. Nanoparticle application to deliver drugs across this barrier is enormously promising. Researchers have reported that nanoparticles can cross several biological barriers for sustained delivery of therapeutic agents for difficult-to-treat diseases like brain tumours (Nazir et al. 2014; Hainfeld et al. 2013).

The potential of nanomedicine with respect to targeted drug delivery has improved with the ease of nanoformulation technique and widened the scope of delivering a range of drugs. Nanomedicine has developed novel diagnostic and screening techniques that have extended the scope of molecular diagnostics. They have been used in vivo to protect the drug entity in the systemic circulation, restrict access of the drug to the chosen sites and deliver the drug at a controlled and sustained rate to the site of action, minimizing undesirable side effects of the drug and allowing for more efficient use of the drug (Bosio et al. 2016).

Today, therapeutic enzymes are considered as one of the most promising applications in the pharmaceutical field. It has been reported by various researchers that enzymatic biocatalyst properties improved considerably by enzyme immobilization on nanomaterials, thereby increasing its stability and reusability and most importantly enhancing their targeting/localization to specific cell and tissues (Golchin et al. 2018; Xin et al. 2017). Gold nanoparticle-based therapeutic biocatalyst provides new tools for the diagnosis and treatment of old and newly emerging pathologies and presents distinctive modality for therapeutic delivery (Table 3.4; Golchin et al. 2018). Thus, gold nanoparticle-based therapeutic enzymes represent a highly promising alternative for treating a variety of pathologies by localized drug delivery approach.

Asparaginase obtained from *Aspergillus terreus* is a potent drug for the treatment of cancer and has antineoplastic or cytotoxic chemotherapy effect (Baskar and

Table 3.4 List of gold nanoparticle-immobilized therapeutic enzymes

Nanocarrier	Therapeutic enzyme/peptide	Type of immobilization method	Application	References
Gold nanoparticle	Asparaginase	Covalent binding method	Anticancerous activity	Baskar et al. (2018)
Gold nanoparticle	Neurotrophin peptides	Adsorption method	Promising drugs in neurodegenerative disorders	Patrizia et al. (2017)
Gold nanoparticle	Glucose oxidase		Biosensing	Muthurasu and Ganesh (2016)
Gold nanoparticle	Superoxide dismutase	Adsorption method	Prevention of oxidative damage from superoxide radicals	Malda et al. (2010)
Gold nanoparticle	Serratiopeptidase	Adsorption method	Strong anti-inflammatory response	Venkatpurwar and Pokharkar (2010)
Gold nanoparticle	Serratiopeptidase	Covalent binding method	Anti-inflammatory activity	Venkatpurwar and Pokharkar (2010)
Silica-coated gold nanoparticles	Oxidase and peroxidase	Adsorption method	Antibacterial properties	Tao et al. (2015)
Gold nanoparticle nanocomposite	Peroxidase	Covalent binding method	Anticancerous activity	Maji et al. (2015)

Renganathan 2012). Malignant cells lack asparagine synthase and employ the free circulating asparagine for its growth. Asparaginase converts the free circulating asparagine into aspartic acid and ammonia thereby lacking the asparaginase and leading to the death of tumour cells. So, research have been carried out across globe to target better asparaginase delivery system by immobilizing asparaginase on to gold nanoparticles followed by procedure of asparaginase gold nano-bioconjugate as potential drug candidate for curbing cancer, by testing against lung cancer cell line and ovarian cancer cell line.

Researchers studied gold nanoparticle-mediated delivery of fungal asparaginase against cancer cells (Baskar et al. 2018). The fungal asparaginase immobilized on gold nanoparticles showed efficient drug delivery in cancer treatment. Fourier transform infrared spectroscopy and nuclear magnetic resonance analysis of the synthesized asparaginase gold nano-bioconjugate showed that primary amines, secondary amines and allylic carbon are the main functional groups concerned with binding of asparaginase onto gold nanoparticles. Increment in the specific enzyme activity of asparaginase was recorded from crude (252.05 U/mg) to gold nano-bioconjugate (364 U/mg). Protein concentration was also increased from 0.018 mg/ml in crude asparaginase to 0.332 mg/ml in gold nano-bioconjugate. Nano-bioconjugate cytotoxicity effect was also observed to be higher against lung cancer cell line A549

than ovarian cancer cell line A2780. Finally, authors demonstrated that synthesized gold asparaginase nano-bioconjugate can be used as an effective anticancer drug and for targeted drug delivery with its increased bioavailability against lung cancer cell line (A549), given that toxicity is 84.51% (Baskar et al. 2018).

Serratiopeptidase, a proteolytic endopeptidase bioenzyme, is recognized as one of the most important therapeutic enzymes having anti-inflammatory activity (Salamone and Wodzinski 1997). Traditionally, therapeutic enzyme delivery is limited due to their poor uptake and vulnerability to degradation inside the gastrointestinal tract. For efficient drug delivery, nanoparticles such as gold nanoparticle complex have immense potential in the therapeutic perspective of biomedicine formulation. With this, the prerequisite is the nanocarrier which plays an important role in the bioavailability of the pharmaceutically active compound, efficiently improving absorption across the gastrointestinal mucosa (Dykman and Khlebtsov 2017).

Venkatpurwar and Pokharkar (2010) have reported the synthesis of gold nanoparticle using a therapeutic enzyme serratiopeptidase at physiological conditions which retained enzyme activity, and serratiopeptidase-capped gold nanoparticle complex led to improved therapeutic benefit. Characterization of synthesized gold nanoparticles has been reported using UV-visible spectroscopy, transmission electron microscopy, X-ray diffraction and Fourier transform infrared spectroscopy. Synthesized nanoparticle stability was assessed at ambient temperature up to 6 months. The retention of enzymatic activity was confirmed by in vitro enzymatic activity and in vivo anti-inflammatory activity of synthesized serratiopeptidase-capped gold nanoparticle complex. The tri-functional role of serratiopeptidase was reported, such as reduction, stabilization and therapeutic activity, finally demonstrating the gold nanoparticles as a nanocarrier for the immobilization and efficient and improved delivery of a therapeutic enzyme for an oral administration with improved therapeutic benefit (Venkatpurwar and Pokharkar 2010).

Tao et al. (2015) studied the bifunctionalized mesoporous silica-supported gold nanoparticles that showed intrinsic oxidase and peroxidase catalytic activities for antibacterial applications for their targeted delivery. Gold nanoparticles have exhibited both oxidase and peroxidase mimicking activities imparting end reactions as reactive oxygen species (ROS). Antibacterial properties proved against both Gram-negative and Gram-positive bacteria.

Superoxide dismutase is an important metalloenzyme and antioxidant defence against free radicals. It catalyses the dismutation of superoxide radicals into hydrogen peroxide and oxygen. Also, catalase is classified under a therapeutic enzymatic group supporting the cell from oxidative damage by reactive oxygen species (Golchin et al. 2018). Reactive oxygen species, such as superoxide radicals, have received great attention due to their involvement in the pathogenesis of various diseases, such as Alzheimer's disease, Down syndrome, cataract, familial amyotrophic lateral sclerosis, Parkinson's disease, cardiac myocytes and several neurological disorders. Superoxide dismutase enzymes have vast physiological importance and therapeutic benefit in the prevention of the oxidative damage from superoxide radicals (He et al. 2013; Zhao et al. 2012). Malda et al. (2010) have synthesized gold

nanoparticle-iron-bound enzyme that demonstrated vast efficacy of gold colloid nanoparticle-bound superoxide dismutase protein.

Maji et al. (2015) have developed the new nanostructured hybrid as a mimetic enzyme for in vitro detection and therapeutic treatment of cancer cells. For targeted drug delivery application in the emerging field of nanobiotechnology, an artificial therapeutic enzyme conjugate was prepared by the immobilization of gold nanoparticles on mesoporous silica-coated nanosized reduced graphene oxide conjugated with folic acid, a cancer cell-targeting ligand. In vitro experiments with bioconjugate hybrid using human cervical cancer cells led to an enhanced cytotoxicity to Henrietta Lacks (HeLa) cells. In the case of normal cells (human embryonic kidney HEK 293 cells), the treatment with the hybrid and H_2O_2 showed no obvious damage, proving selective killing effect of the hybrid to cancer cells. Hybrid therapeutic enzyme bioconjugate with peroxidase activity has dual applications: firstly, detection (selective quantitation and colorimetric) of cancer cells and, secondly, cancer therapy by activating oxidative stress. Both detection and therapeutic processes are selective to cancer cells, indicating high specificity and robustness of the hybrid (gold nanoparticle) conjugate proved as a promising candidate for clinical cancer diagnostics and treatment and their targeted drug delivery approach (Nasrabadi et al. 2016).

It can be inferred from few of the above-discussed studies of nanocarrier-bound therapeutic enzyme delivery that nanocarrier-based approach such as gold nanoparticle-immobilized enzymes represents an important modality within therapeutic and diagnostic biomedical applications including cancer, cardiovascular diseases and brain diseases.

3.6 Conclusion

Gold nanoparticles offer an excellent platform for biomedical applications due to their unique physical and chemical properties. Amongst the various physicochemical and biological methods of gold nanoparticle syntheses, the biological route has become most fascinating due to total avoidance of toxic chemical and ambient reaction conditions and more biocompatibity of the gold nanoparticles, since delivery of enzyme as drug along with the antimicrobial property of gold nanocarrier adds additional double effects on various health ailments. Very few therapeutic microbial enzymes are used till date, and more research on gold nanocarrier-bound therapeutic important enzyme is the need of the hour.

The selection of the most appropriate methods for robust gold nanocarrier design needs a thorough understanding of non-covalent and covalent interactions at the interface of different types of therapeutic enzymes and different functionalized gold nanoparticles. It is inferred that gold nanocarrier-bound limited therapeutic enzyme has shown promising results in the treatment of central nervous system disorders. To sum up, gold nanocarrier-mediated delivery of therapeutic enzymes holds a great potential for biomedical applications.

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Chapter 4
Improving Bioavailability of Vitamin A in Food by Encapsulation: An Update

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Contents

Abstract Vitamin A is an obligatory micronutrient for healthy human life as it cannot be synthesized de novo and has to be acquired from dietary sources. The poor water solubility and susceptibility against photochemical degradation make vitamin A relatively unstable during food processing as well as storage. To combat prevailing vitamin A deficiency, various strategies have already been adopted in pharmaceutical industries to develop vitamin A formulation which has the ability to protect

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and minimize its degradation. On the one hand, in pharmaceutical formulations, vitamin A may be coupled with sub-toxic effects due to its buildup in the liver and other vital organ, while on the other hand its involvement against various health disorders such as neurodegenerative diseases, cardiovascular diseases and cancer has recently compelled the population to achieve vitamin A via pharmaceutical supplements, functional foods or food supplements. The success of pharmaceutical application encouraged food technologists to develop numerous premixes encapsulating vitamin A appropriately which can be successfully applied for the development of food supplements or vitamin A-rich functional foods. So this chapter is an update of the principal encapsulation techniques adopted for the development of vitamin A nanomaterials to improve its bioavailability and associated challenges with fabrication method.

Keywords Vitamin A · Retinol · Encapsulation · Bioavailability · Micro-/nanoencapsulation · Toxicity · Functional food

Abbreviations

ABCA	ATP-binding cassette
AFM	Atomic forces microscopy
ATP	Adenosine triphosphate
CRBP	Cellular retinol binding protein
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
EU	European Union
FDA	Food and Drug Administration
FSSAI	Food Safety and Standards Authority of India
FTIR	Fourier transform infrared
GIT	Gastrointestinal tract
HSCs	Hepatic stellate cells
LRAT	Lecithin retinol acyltransferase
nm	Nanometer
O/W	Oil in water
O/W/O	Oil-in-water-in-oil
RDA	Recommended dietary allowance
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
W/O	Water in oil

W/O/O	Water-in-oil-in-oil
W/O/O/W	Water-in-oil-in-oil-in-water
W/O/W	Water-in-oil-in-water
WHO	World Health Organization
μg/d	Microgram/day

4.1 Introduction

The role of vitamin A in vision health is well celebrated in literature. Further, it is also observed that the role of vitamin A is not only limited to vision but it also participates in various physiological functions. Today, vitamin A deficiency is prevailing across the globe; hence it is reasonable to address vitamin A with respect to its history, chemistry, role in human health and innovative techniques improving vitamin A stability in the food system.

In view of chemistry, all-trans-retinol and its derivatives possess an electron dense section which attracts electron-deficient species particularly free radicals (Krinsky and Johnson 2005; Mueller and Boehm 2011). Hence these retinoids are prone to oxidation in the presence of oxidants, transition metals, free-radical-generating agents as a consequence of its isomerization (at 9, 11 and 13 positions) or/and oxidative degradation. It is also evident that retinoids are susceptible to thermal stress resulting in heat-induced isomerization hence creating 13-cis isomers (Panfili et al. 1998). The degradation of vitamin A in an aqueous media is a well-known fact. Likewise, due to its low polarity, vitamin A displays poor solubility in aqueous media. The isomerization and degradation result in partial or complete loss of its activity. The structure of compounds displaying vitamin A activity is recalled in Fig. 4.1: (A) natural retinoids and (B) synthetic retinoids.

4.1.1 Absorption, Transport, and Metabolism

Accruing evidence on the involvement of vitamin A in various health disorders encouraged more investigations focusing its metabolism which was found to be a complex signalling pathway. In general, vitamin A precursors or derivatives (retinol esters present in food of animal origin) are absorbed by the brush-bordered enterocytes in the small intestine as a consequence of enzymatic hydrolysis of retinyl ester into retinol. The mechanism through which retinol enters in enterocytes is still unclear. Further within enterocytes, retinol binds to cellular retinol-binding protein type II (CRBP) as well as lecithin retinol acyltransferase (LRAT), which are responsible for its de novo esterification. Furthermore, these newly catalyzed retinyl ester molecules are integrated within the chylomicron and excreted into the lymphatic circulation system, which comprises about 20–60% of total retinol efflux from brush-bordered enterocytes, while non-esterified retinol molecule enters in the por-

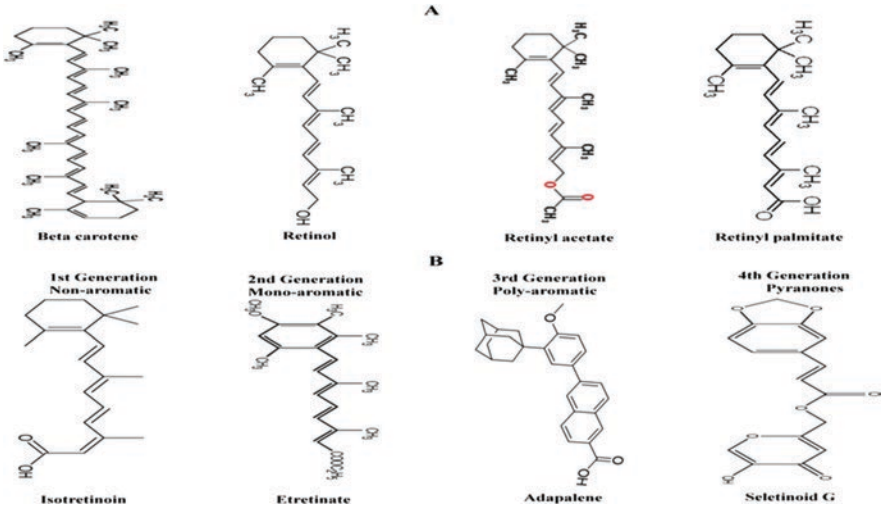

Fig. 4.1 (**A**) Structure of some natural retinoids. (**B**) Structure of some synthetic retinoids

tal vein via ATP-binding cassette (ABCA1) transporter, which is responsible for only 30% of total retinol intake. Chylomicrons incorporating retinol are engulfed by hepatocyte cells in the liver and then transported to hepatic stellate cells (HSCs) and stored in lipid globules. The mechanism via which these vitamin A molecules are stored and released is not clearly understood.

An array of factors monitors its uptake in the human gastrointestinal tract (GIT) when it travels along with foods. These factors include variations in its physiochemical form (carotenoid species, their physiological linkages and vitamin A activity), the complexity of food matrices (variety and quantity of fatty acids, dietary fibres, doses of retinoids, location of retinoids in animal as well as plant tissue, processing condition and size of food particles, absence/presence of retinoid enhancer and inhibitor) and interaction among retinoids and fat-soluble nutrients and host-associated factors (surgery, age, disease, fed condition, obesity and genetic variation).

4.1.2 Source and Intake

With the lack of ability for de novo synthesis, the human is compelled to consume vitamin A from dietary sources in order to meet the recommended daily allowance. The total dietary vitamin A includes carotenoids from plant origin, retinol and its ester from animal origin or supplements and functional foods and synthetic reti-

noids from pharmaceutical supplements (Fig. 4.1). Beta carotene displays the most potent vitamin A activity among the naturally occurring carotenoids (α-carotene and β-cryptoxanthin) and is generally found in carrot, sweet potato, pumpkin and green leafy plants. In industrialized countries, 25–75% of total dietary vitamin A is met via preformed vitamin A via food or supplements, while the rest of the proportion of vitamin A is obtained by carotenoids. In general pharmaceutical, supplements contain a high dose of vitamin A (synthetic or natural retinoids) and its frequent consumption may lead to hypervitaminosis.

The recommended dietary allowance (RDA) may vary depending upon the age, sex and biological cycles such as 400–600 μg/d (children of age 1–8 years), 600–800 μg/d (children above 8 years old), 900 μg/d (adult men) and 800 μg/d (adult women) (Council 1989; Olson 1987). Conversely, adult women during pregnancy are allowed to consume 700 μg/d of the vitamin A to avoid teratogenic effects on the foetus (Chapman 2012; Rothman et al. 1996).

4.1.3 Deficiency and Toxicity

In the current scenario, approximately 2 billion of the world population is affected by micronutrient deficiencies and the major contribution is the dietary deficiency of minerals and vitamins. Further, 254 million preschool-aged children are found to be vitamin A deficient. As per the WHO mortality report, approximately 1.5% of total deaths (0.8 million deaths) is endorsed by vitamin A deficiency (WHO 2009). In addition, it was estimated that 19.8 million pregnant women suffer from low vitamin serum level (<1.05 μmol L-1) and 6.2 million of those are affected with gestational night blindness (West 2002). Further, it was estimated that two-thirds of vitamin A-deficient women live in South and Southeast Asia.

Various strategies have been adopted to address vitamin A deficiency such as pharmaceutical supplements and fortification of staple foods such as rice and cereal flour. Frequent ingestion or a single high dose of vitamin A supplements results in undesirable effects. Various reports have been documented addressing the acute toxicity of vitamin A (Bauernfeind 1980). The acute toxicity is generally attributed to bad food choice as well as frequent consumption of high-dose supplements. Anoxia, blurred vision, nausea, vomiting, bone pain and headaches are some symptoms of acute toxicity. Today, acute toxicity of vitamin A is uncommon phenomenon due to various reasons: modern diet system does not comprise of a high dose of vitamin A, prescription of pharmaceutical supplements is well regulated, and supplementation programs are well regulated and supervised. In spite of effective regulation, some cases of acute intoxication were documented which were mainly due to consumption of a high dose of supplements due to unawareness (Allen and Haskell 2002; Penniston and Tanumihardjo 2006).

4.1.4 Consequence of Deficiency

Vitamin A deficiency is recognized as one of the most prevailing micronutrient malnutrition disorder and can be easily reversed with its adequate supply. Various reports clearly suggest its involvement in broad-spectrum physiological functions apart from vision. Hence its insufficient intake may result in loss of vision, xerophthalmia, corneal xerosis, corneal ulceration, corneal necrosis and blindness. The importance of vitamin A goes beyond vision such as spermatogenesis, reproduction, embryonic development, cellular growth, cellular differentiation, maintenance of epithelium, impaired immunological function, anaemia, skin disorders (psoriasis and acne), neurological disorders (schizophrenia and Alzheimer's disease), cancers, infection, morbidity and mortality.

4.2 Improving Vitamin A Bioavailability Through Nanotechnology

Nanotechnology is exploited by food technologists to develop a range of carrier systems for the encapsulation, protection and controlled release of vitamins. Vitamin A cannot directly be incorporated into the food matrix in their pure form due to biological and physicochemical constraints (Yao et al. 2014). These factors involve poor solubility (in oil and/or water) and susceptibility to physicochemical, photochemical and enzymatic degradation during processing, transport and storage. These challenges have encouraged food technologists to design food-grade nanomaterials with many advantages: (i) improved thermodynamic or kinetic stability that gives significantly greater stability over direct incorporation; (ii) facilitate encapsulation of hydrophilic as well as lipophilic micronutrient; (iii) improved bioavailability of micronutrient due to its small droplet size, higher surface area and better absorption in the GIT; and (iv) enhance bioavailability by minimizing first-pass metabolism.

The bioavailability of fat-soluble vitamins can be defined as the part of the consumed vitamin that finally ends in the systemic (blood) circulation as an active form. For ingested vitamin, there are several factors which inhibit it from reaching the systemic circulation in an active form, e.g. chemical instability during the digestion process, poor solubility in the gastrointestinal tract (GIT) fluids, slow uptake in the GIT and first-pass metabolism. To design efficient nanomaterials for vitamin A, it is indispensable to understand the biological processes that monitor the absorption of vitamin A and bioavailability. The schematic of Fig. 4.2 depicts some of the main processes integrated with the uptake of vitamin A encapsulating nanomaterials. After ingestion, food is partially digested (mainly by mastication) in the oral cavity. Further, this partially digested food passes through the stomach (pH ~ 1 to 2) for enzymatic actions that facilitate the release of vitamin A from the food matrix.

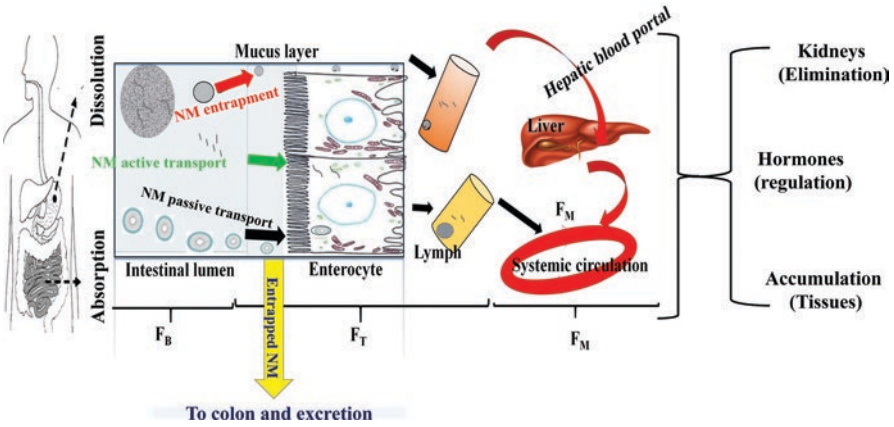

Fig. 4.2 The fate of encapsulated vitamin A in intestinal lumen. $\mathbf{F_B}$: fraction of the encapsulated vitamin A which is released from food matrix into the gastric juice in GIT. $\mathbf{F_A}$: fraction of the vitamin A which is transported through the intestinal epithelium and then transported to the portal or lymph. $\mathbf{F_M}$: The fraction of the absorbed vitamin A which is an active form after bypassing the chemical modification by organs such as liver and kidney. NM: nanomaterials

These digestive enzymatic actions are the major stakeholder in metabolic loss of vitamins, known as the first-pass metabolism. It was hypothesized that vitamin A encapsulated in nanomaterials may have higher bioavailability than that of the free vitamin. This speculation came to end with a dedicated study where researcher witnessed high bioavailability of carotenoids when ingested in emulsified form (incorporated within nanomaterials) than that of pure carotenoids (Faulks and Southon 2005). The bioavailability of vitamin A can further be improved with nanomaterials which can bypass liver metabolism (Yao et al. 2015). Further, the bioavailability of vitamin A can also be improved by nanomaterials which can facilitate paracellular transport of vitamin by altering the integrity of tight junctions of nanoparticles. It is believed that paracellularly transported lipophilic compounds are not exposed to metabolic activity of intracellular enterocyte enzymes and may, therefore, have higher bioavailability (Yao et al. 2015). Hence, the oral bioavailability (F) of encapsulated vitamin A in nanoparticles can be determined by the following equation:

$$F = F_B * F_A * F_M$$

Here, F_B is the portion of ingested vitamin A that survived through the upper GIT and is released from the food matrix/nanoparticles into the GIT, thus becoming bioavailable for absorption by enterocytes. F_A is the portion of the bioavailable vitamin, which is ultimately absorbed by the enterocytes and then reached the portal blood or lymph systemic circulation. F_M is the portion of absorbed vitamin A which survives in its active form after the first-pass metabolism in the GIT and liver (and any other forms of metabolism) (Maurya and Aggarwal 2017; Yao et al. 2015).

4.2.1 Improving Bioavailability Through Nanomaterials

The first-pass metabolism is accountable for reduced oral bioavailability as it causes degradation of most of the ingested drugs, resulting in a fraction of ingested drug reaching the systemic circulation in the active form. Nanomaterials encapsulating vitamin A can bypass the first-pass metabolism resulting in improved bioavailability.

4.3 Encapsulation

Currently, a broad spectrum of foods are being supplemented with vitamin A but its direct addition in target food may cause inevitable interactions which may lead to the compromise in food appearance, taste, quality and its bioavailability, resulting in a drastic reduction in its efficacy as the disease-combating compound. These limitations challenged food technologists to come up with innovative techniques which not only ensure high bioavailability but also evade undesirable interaction and do not influence the customer acceptability.

The success of encapsulation of vitamin A in pharmaceutical formulation attracted food technologists to encapsulate vitamin A food application with the following objectives: (i) beat solubility obstacle between vitamin A and the food matrix; (ii) protect vitamin A against physiochemical stress such as pH, temperature, moisture and oxidation; (iii) promise improved bioavailability with the controlled and site-specific release of encapsulated vitamin A; (iv) to not influence appearance, taste and quality of food matrices, hence maintaining customer acceptability.

4.3.1 Principal Encapsulation Techniques

Literature reports about the availability of a broad spectrum of encapsulation techniques for bioactive compounds which are discussed and reviewed addressing their physiochemical attributes such as wall materials, size and shape of microparticles (Gonçalves et al. 2016; Katouzian and Jafari 2016; Sauvant et al. 2012). Various researchers have addressed generally recognized as safe wall material in their excellent books and critical review addressing. However, the correlation of listed data on wall material specific to vitamin A encapsulation is limited. Various polymers have been used for vitamin A encapsulation from wall materials, as listed in Table 4.1. In this section, we will discuss principal techniques applied exclusively for vitamin A encapsulation (Table 4.2).

Table 4.1 Wall materials generally recognized as safe suitable for microencapsulation in the food industry

Class of wall materials	Origin	Subclass	Names
Carbohydrate polymers	Plant	Starch and derivative	Amylose Amylopectin Maltodextrins Dextrins Polydextrose
		Cellulose and derivatives	Methylcellulose Hydroxypropyl methyl cellulose Hydroxypropyl cellulose Ethyl methylcellulose Ethylcellulose
		Plant exudates	Gum arabic Gum karaya Mesquite gum Pectins
		Plant extract	Galactomannans Soluble soybean
	Marine		Carrageenan Alginate
	Microbial and animal		Xanthan Gellan Dextran Chitosan Cyclodextrin
Protein	Plant	Protein and derivates	Gluten (corn) Isolates (pea, soy)
	Microbial and animal	Protein and derivates	Caseins Whey proteins Gelatin
Lipid	Plant	Fatty acids/alcohols Glycerides Waxes Phospholipids	Beeswax Carnauba wax Candelilla wax
	Microbial and animal	Fatty acids/alcohols Glycerides Waxes Phospholipids (shellac)	Milk phospholipid Fish oil

Spray-Drying

Spray-drying is one of the oldest techniques used to encapsulate bioactive compounds. The bioactive compounds need to be solubilized in the dispersion containing wall material to obtain a homogenized system. Then the homogenized system is fed to the spray-dryer and atomized by a hot air which leads to the formation of a microparticle as a result of water evaporation (Fig. 4.3). The encapsulation process

Table 4.2 Encapsulation techniques adopted for encapsulation of vitamin A

Nanomaterials	Composition	Preparation methods	References
Spray-dried powder	Gelatin, sucrose, peach gum	Spray-drying	Xie et al. (2006)
	Starch octenylsuccinate, OSA-starch	Spray-drying	Xie et al. (2010a)
	Starch octenylsuccinate	Spray-drying	Xie et al. (2007)
	B-Lactoglobulin	Spray-drying	Liu (2003)
	Arabic gum	Spray-drying	Gonçalves et al. (2017)
Spray-cooled powder	Palm hydrogenated oil	Spray-cooling	Wegmüller et al. (2006)
	Hydrogenated oil	Spray-cooling	Zimmermann et al. (2004)
Emulsion system	Polyoxyethylene hydrogenated castor oil, polyoxyethylenediisostearate, polyoxyethylenedioleate	Emulsification	Yoshida et al. (1999)
	Distearoylphosphatidylcholine, cholesterol	Solvent evaporation	Kawakami et al. (2005)
	N-Trimethyl chitosan, lecithin, Cholesterol	High-pressure homogenization	He et al. (2013)
	Casein	High-pressure homogenization	Mohan (2014)
	Chitosan	Emulsification	Pisetpackdeekul et al. (2016)
	Sodium caseinate, Polysorbate 80	Emulsification	Loewen (2014)
	Compritol1 ATO 888, Span 80	Homogenization	Clares et al. (2014)
	Tween 20, Canola oil	Homogenization	Chaudhari and Nitin (2015)
	Sodium caseinate, zein	Microfluidization	Pan et al. (2015)
	Saponin	Emulsification	Choudhry et al. (2016)
	Whey protein isolate	High-pressure homogenation	Beaulieu et al. (2002)
	Cremophor EL, soybean oil, hydroxyl propyl methyl cellulose	Emulsification	Taha et al. (2004)
	Silica nanoparticles, caprylic/capric triglyceride, soybean lecithin	High-pressure homogenization	Eskandar et al. (2009)
	Lecithin, Miglyol®812	High-pressure homogenization	Ghouchi-Eskandar et al. (2012)
	Polylactic acid	Single emulsion technique	Puntel et al. (2015)

Liposome	Dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine	Dehydration/rehydradation	Lee et al. (2003)
	Soybean phosphatidylcholine, cholesterol	Dehydration/rehydration method	Lee et al. (2005)
	Distearoyl-L-phosphatidylcholine, cholesterol	Solvent evaporation/rehydration	Siddikuzzaman and Grace (2013)
	Lecithin Cholesterol	Film hydration/sonication method	Pezeshky et al. (2016)
	Phospholipids	Thin-film hydration method	Clares et al. (2014)
	Phosphatidyl choline	Solvent evaporation and rehydration method	Lee et al. (2002)
	L-phosphatidylcholine	Dehydration/rehydration	Ko and Lee (2010)
	N-Trimethyl chitosan	Film dispersion method	Wen et al. (2010)
	Distearoylphosphatidylcholine, cholesterol	Dehydration/rehydration	Siddikuzzaman and Grace (2012)
	Distearoyl-L-phosphatidylcholine, cholesterol	Film hydration and sonication	Siddikuzzaman and Grace (Siddikuzzaman and VMB 2014)
	Distearoyl-L-phosphatidylcholine, cholesterol	Film hydration	Berlin Grace and Rimashree (2015)
Solid lipid nanoparticles	Compritol 888 ATO, caprylic/capric triglycerides	Hot homogenization	Jenning et al. (2000a)
	Compritol 888 ATO, caprylic/capric triglycerides	Hot homogenization	Jenning et al. (2000b)
	Compritol 888 ATO	Hot homogenization	Jenning et al. (2000b)
	Cetylpalmitate, caprylic/capric triglycerides, polyglyceryl-3 methylglucosedistearate	Hot homogenization	Jung et al. (2013)
	Caprol PGE-860), soybean lecithin	High-pressure homogenization	Xia and Kong (2011)
	Cetylpalmitate, glyceryl behenate, palmitic acid	Hot homogenization	Carlotti et al. (2005)
	Cyclodextrins	Solvent evaporation	Semenova et al. (2002)
	Precirol ATO5, Pluronic F68	Hot homogenization	Carafa et al. (2008)
	Gelucire 44/14® (G), tween 80®	High-pressure homogenization	Argimón et al. (2017)
	Labrafac lipophile, Labrasol, Plurol oleique	Sonication	Clares et al. (2014)
	Witepsol, Precirol ATO	Hot homogenization	Cerreto et al. (2011)

(continued)

Table 4.2 (continued)

Nanomaterials	Composition	Preparation methods	References
Molecular complexes	B-Cyclodextrins	Freeze-drying	Vilanova and Solans (2015)
	Cyclodextrin	Precipitation	Braithwaite et al. (2017)
	Modified sodium caseinate	Stirring	Gupta et al. (2018)
	B-Lactoglobulin	Molecular complexation	탕가문 (2015)
	Whey protein isolate	Hydration	Herath (2007)
	Succinated chitosan	Homogenization	Huang et al. (2013)
	Pectin	Co-solvent desolation	Suh et al. (2014)
	B-Lactoglobulin	Air bath oscillation	Tang et al. (2017)
	Ovalbumin	Heating	Visentini et al. (2017b)
	Native ovalbumin, high methoxyl pectin	Heating	Visentini et al. (2017a)
	PLA	Nanoprecipitation	Yildirim et al. (2017)
	Casein	Stirring	Loewen et al. (2018)
	Pectin, tween 80	Microfluidization	Noh et al. (2018)
Electrospinning	Cellulose acetate	Electrospinning	Taepaiboon et al. (2007)
	Poly(ethylene glycol), $CaCl_2$, poly(d,L-lactide)	Electrospinning	Müller et al. (2015)

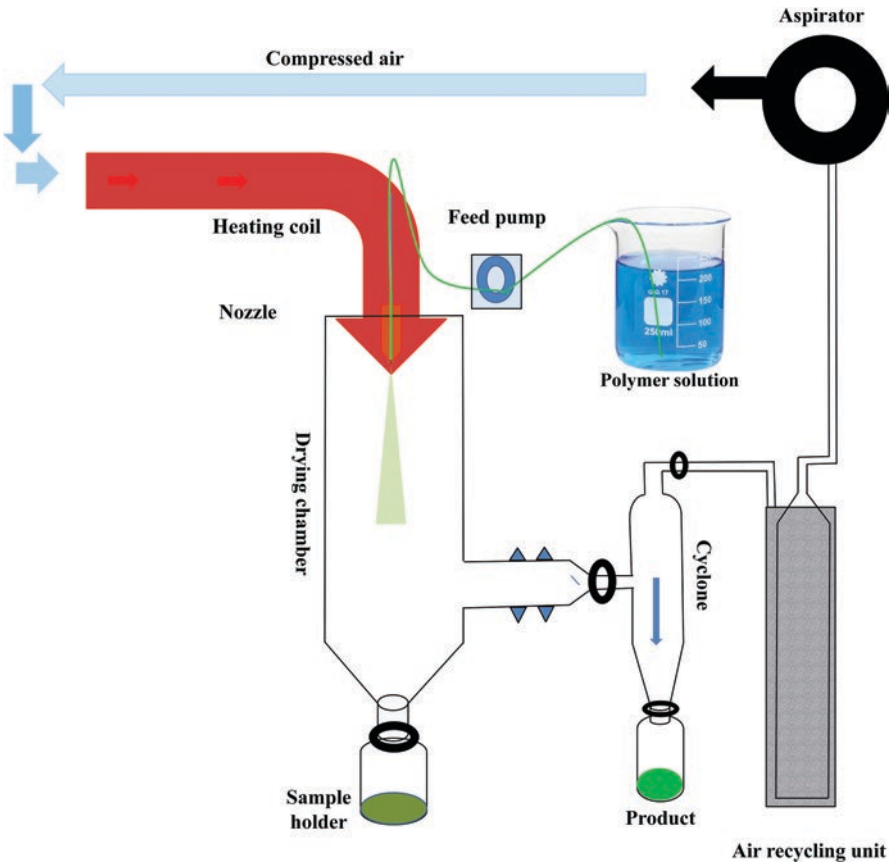

Fig. 4.3 Schematic diagram of vitamin A encapsulation using spray-drying process

is influenced by an array of factors such as homogeneity of the dispersion system, amount and type of emulsifier applied, the viscosity of the dispersion system, feed rate, inlet and outlet temperature and flow rate of hot air. Despite great flexibility, better control on microparticle size and shape, continuous and reproducible, low cost and easy scale-up, spray-drying was adopted for vitamin A after 20 years (1950) of its development (1930) (Desai and Jin Park 2005; Schafroth et al. 2012; Xie et al. 2010b; Xie et al. 2006). Further, the application of spray-drying for vitamin A encapsulation is even rarer as it requires the bioactive agents in water-soluble form (Desai and Jin Park 2005; Gonçalves et al. 2016; Katouzian and Jafari 2016; Sauvant et al. 2012; Xie et al. 2010a; Xie et al. 2007). Spray-drying provides great flexibility in selection of wall materials individually or in combination. Despite various advantages, spray-drying remained untapped for vitamin A encapsulation which can be attributed to resultant porous microparticle which could be susceptible to degradation of encapsulated vitamin A. The use of spray-drying for vitamin A encapsulation also carries several limitations such as low encapsulation efficiency,

premature release, degradation of carrier oil required to dissolve vitamin and involvement of high temperature. These factors pose several challenges for engineers to design a spray-dryer which can address these issues, hence limiting the mass-scale production of the nanoparticles encapsulating vitamin A (Okuro et al. 2013). To overcome these limitations, the application of spray-drying in combination with other encapsulation methods can be suitable for getting vitamin A encapsulated nanomaterials with improved properties.

Spray-Cooling/Spray-Chilling

These techniques are very similar to microencapsulation by spray-drying and involve diffusion of vitamin A in a molten lipid (Fig. 4.4). However, the dispersion is atomized through heated nozzles by cooled air. These techniques were found to be a suitable process for encapsulating heat-sensitive fat-soluble vitamins. Only a

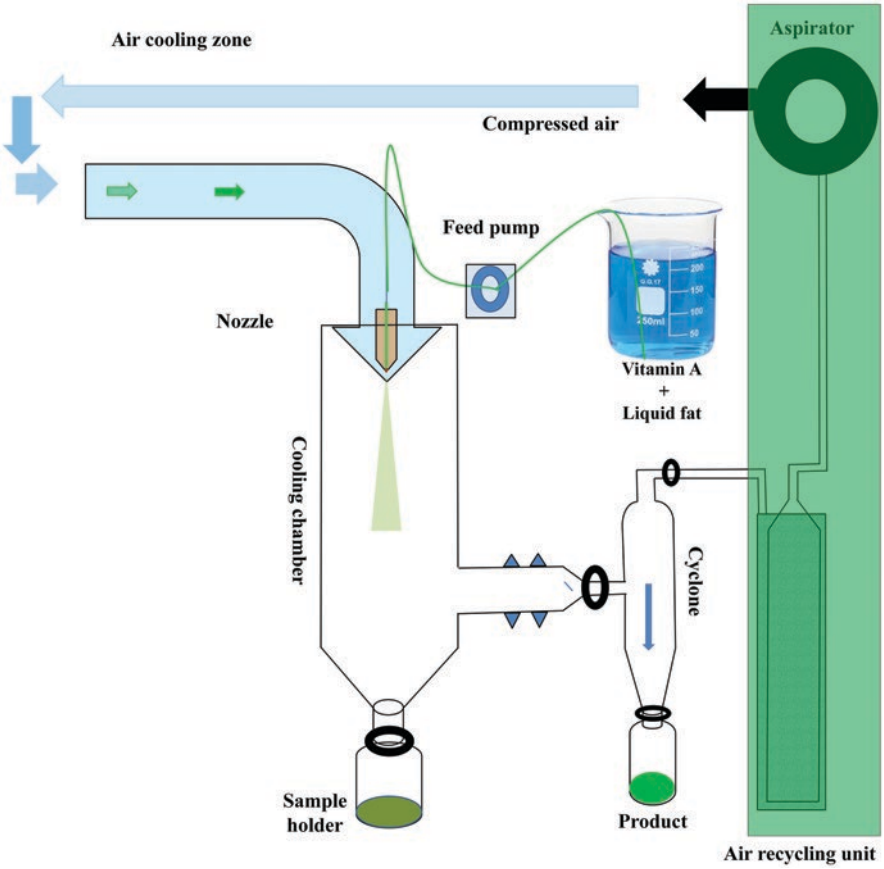

Fig. 4.4 Schematic diagram of vitamin A encapsulation using spray-chilling/cooling process

single report is produced addressing vitamin A encapsulation by spray-cooling methods where iodine, iron and vitamin A were encapsulated in microparticles which displayed excellent retention of the encapsulated vitamin during 6 months of the storage period (Wegmüller et al. 2006). Maintenance of uniform quality of resultant nanomaterials is a challenge to engineers which make spray-cooling/spray-chilling hard to scale up for mass production for nanomaterial encapsulating vitamin A. Further, the nanomaterials prepared by spray-chilling are generally insoluble in water due to the carrier oil used to dissolve vitamin A, which limits its food application. Further, it is also noticed that the matrix of nanomaterials obtained from spray-chilling is efficient in encasing bioactive core ingredient.

Emulsion System

This process is comprised of at least two immiscible phases (lipid and water) where one phase is dispersed as small spherical droplets within another phase (Fig. 4.5). Depending on the spatial arrangement of the two phases, the emulsion is generally categorized into two groups, i.e. oil in water (O/W) or water in oil (W/O). These two immiscible phases are stabilized by various surfactants and emulsifiers (Loveday and Singh 2008). Further literature also reports about more complex emulsion systems such as oil-in-water-in-oil (O/W/O), water-in-oil-in-water (W/O/W), water-in-oil-in-oil (W/O/O) or water-in-oil-in-oil-in-water (W/O/O/W) (Gao et al. 2010; Lee et al. 2001; Zheng 2009). Literature reports that the emulsion system is one of the most adopted techniques for encapsulation of vitamin A, but before selection of the kind of emulsion system, various factors need to be taken into consideration such as quantity and type of carrier oils and surfactant and absence/presence of antioxidants. It was noticed that the chemical stability of encapsulated vitamin A solely

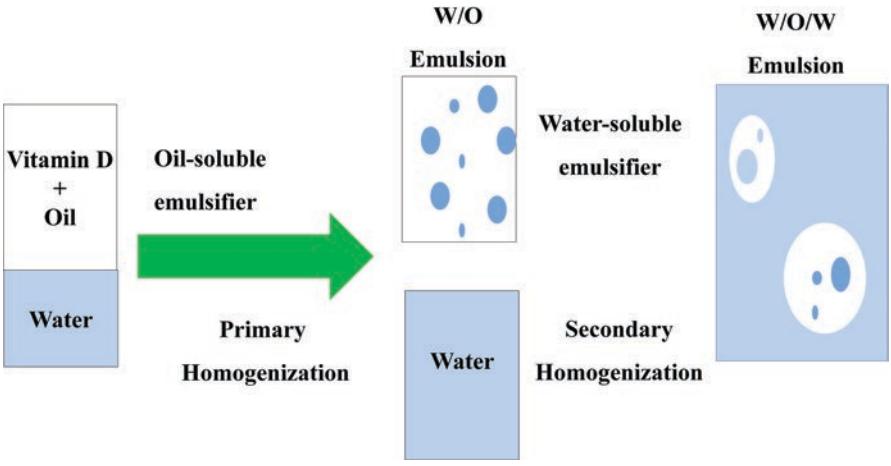

Fig. 4.5 Schematic diagram of the emulsion preparation where W/O and W/O/W represent water-in-oil emulsion and water-in-oil-in-water emulsion

depends on the stability of the emulsion system. Further, Yoshida compared the ability of different emulsion systems in retaining encapsulated nanoparticles and stability of retinol in various emulsion systems was found in the following order: O/W/O > W/O > O/W (Yoshida et al. 1999). Further, Yaniki observed that O/W/O emulsion system containing retinol provides stability during 1-month storage period (Yanaki 2001). Conversely, it was also observed that the presence of an antioxidant in the emulsion system also improves the stability of vitamin A in the emulsion system (Lee et al. 2004; Moyano and Segall 2011).

Liposome

The literature describes various preparation methods for liposome (Bozzuto and Molinari 2015; Daeihamed et al. 2017; Haghiralsadat et al. 2017; Huang et al. 2014; Kim 2016; Liu et al. 2015; Mozafari et al. 2008; Nekkanti et al. 2015; Sagalowicz and Leser 2010; Wakaskar 2017). Generally, liposomes are spherical liquid structures with an aqueous core surrounded by a single (unilamellar liposomes) or multiple lipid bilayers (multilamellar liposomes) (Fig. 4.6). Based on size, it is also categorized as nanoliposome (≤200 nm). The ability to host both hydrophilic and hydrophobic bioactive agents individually or simultaneously makes liposomes the most celebrated encapsulation technique for fat-soluble compounds. Apart from flexibility in size and composition, liposome also offers high biocompatibility with animal tissue as it exhibits a resemblance to the natural cell membrane. It is also well tested for encapsulating vitamins (Arsić and Vuleta 1999; Breusch and Rager 2004; Cristiano et al. 2017; He et al. 2013; Hwang and Ludescher 2002; Kawakami et al. 2005; Lee et al. 2003; Lee et al. 2005; McCormack and Gregoriadis 1998; Pezeshky et al. 2016; Redmond et al. 2007; Sagalowicz and Leser 2010; Siddikuzzaman and Grace 2013; Singh and Das 1998). Though, vitamin A demonstrated high chemical stability when it is incorporated within liposome, its application in food fortification is still untapped. In addition to this, a commercial form of vitamin A-containing liposome is presently available in the market (Keller 2001).

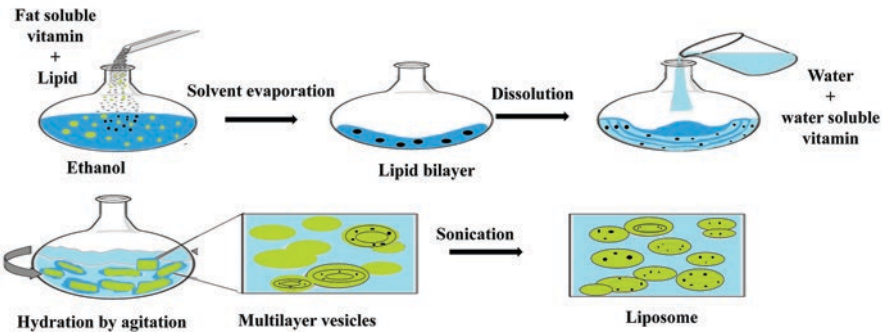

Fig. 4.6 Schematic diagram of the vitamin A encapsulation using liposome method

Solid Lipid Nanoparticles

Solid lipid nanoparticle is the most suitable means for vitamin encapsulation as it is the hybrid structure of the liposome and emulsion system, hence offering an array of benefits such as high drug loading capacity, greater encapsulation efficiency and greater chemical stability against physiochemical stress. Literature reports various preparation methods for solid lipid nanoparticles (Aditya and Ko 2015; Gao and McClements 2016; Geszke-Moritz and Moritz 2016; Gobbi de Lima et al. 2016; Haghiralsadat et al. 2017; Naseri et al. 2015; Nik et al. 2012; Sharma 2016; Wakaskar 2017; Weber et al. 2014; Yadav et al. 2013). Similarly, the ability of solid lipid nanoparticles to encapsulate and protect vitamin A is documented in various studies where vitamin A was incorporated in solid lipid nanoparticles to evaluate the chemical stability of entrapped vitamin A against different physiochemical stress (Carlotti et al. 2005; Jee et al. 2006; Jenning and Gohla 2001; Jenning et al. 2000a; Jenning et al. 2000b; Jung et al. 2013; Pan et al. 2016; Sapino et al. 2005; Toriyabe et al. 2017; Xia and Kong 2011). Further, retinyl palmitate encapsulated in solid lipid nanoparticles demonstrated higher protection (51% and 54% after 120 min) under UVA and UVB exposure (Carlotti et al. 2005). Similarly, 43% higher photostability of all-trans retinol in methanol solution was noticed when it was encapsulated in solid lipid nanoparticles (Jee et al. 2006). Further, retinyl palmitate showed higher (204 days) half-life (t_{50} value) at 40 °C when it was encapsulated in solid lipid nanoparticles with glyceryl behenate matrix (Jenning and Gohla 2001). Similarly fourfold higher retention was witnessed in the gel having retinyl palmitate in solid lipid nanoparticles over 120 days than that of the gel with free retinyl palmitate (Sapino et al. 2005).

Molecular Complex

Molecular complex is generally performed by application of cyclodextrin which can host bioactive agents within its cavity. Cyclodextrin is widely used for encapsulation of vitamin A in pharmaceutical formulations to evaluate its chemical stability against various physiochemical stresses (Braithwaite et al. 2017; Jarho et al. 1996; Koeda et al. 2014; Lin et al. 2007; Lin et al. 2000; McCormack and Gregoriadis 1998; Moldenhauer et al. 1999; Munoz-Botella et al. 2002; Semenova et al. 2002; Trichard et al. 2007; Vilanova and Solans 2015; Yap et al. 2005).

Electro-spinning

The electrospinning technique is a highly versatile technique which is a combination of two techniques, namely, electrospray and spinning. It involves the application of a high voltage to the droplet of fluid (melt or solution) coming out from the syringe of a die on a spinning disc which acts as one of the electrodes (Fig. 4.7). The solvent evaporates when the droplets travel and nanofibers obtained from their

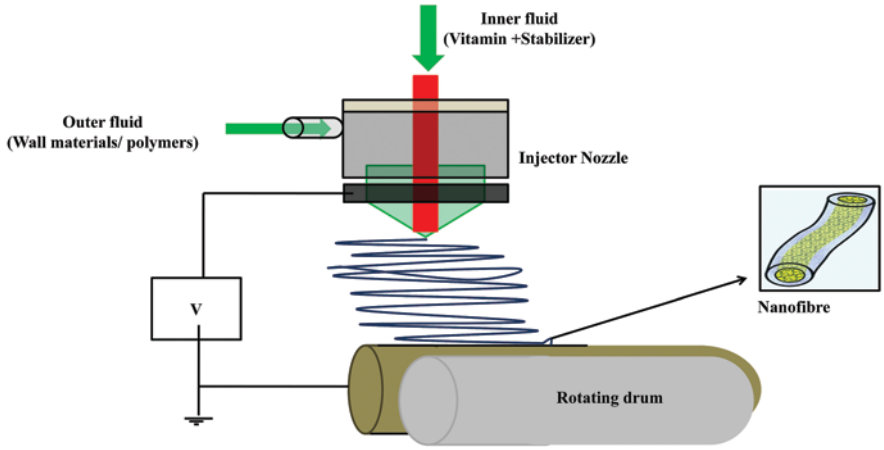

Fig. 4.7 Schematic diagram of the electrospinning technique process for vitamin A encapsulation

process are far from thermodynamic equilibrium. The produced droplet will be distorted by centrifugal force and shaped into a cone structure which is finally converted into a thin fibre-like structure (nanofiber). Various researchers have critically reviewed electrospinning with respect to fundamental, principle, type and food application (Gómez-Mascaraque et al. 2018; Wen et al. 2017). Literature reports that electrospinning is one of the widely adopted encapsulation technique for thermosensitive bioactive agents but its application in encapsulating vitamin A is very limited. To date, only two reports are documented which facilitated vitamin A encapsulation (Ghorani and Tucker 2015; Müller et al. 2015; Taepaiboon et al. 2007). The prime reason for its limited application is attributed to its requirement of synthetic polymers rather than on biopolymers. Second, the low throughput of the electrospinning system also drags its commercial exploitation at a large scale. Finally, the optimization of encapsulation process with food grade polymers and fat-soluble vitamin is hard to optimize with electrospinning due to their poor viscoelastic behaviour, poor solubility, lack of sufficient molecular entanglement and few operation parameters which are hard to control (Ghorani and Tucker 2015).

4.3.2 Characterization Techniques for Encapsulated Structures

Literature reports about various techniques which are adapted for characterization of encapsulated nanoparticles. Atomic force microscopy (AFM), dynamic light scattering (DLS) (Carafa et al. 2008; Lee et al. 2003; Pan et al. 2016), cryo-transmission electron microscopy (TEM) (Mohan 2014), scanning electron microscopy (SEM) and transmission electron microscopy (Gupta et al. 2018;

Numata et al. 2015; Pisetpackdeekul et al. 2016) are most widely used in most of the studies to determine the particle size and distribution of vitamin A nanoparticles. The surface charge on nanoparticles is another crucial factor for determining the stability of nanoparticles. Generally, the surface charge is evaluated in terms of zeta potential, which is determined by laser Doppler anemometry. Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (Argimón et al. 2017; Jenning et al. 2000b; Müller et al. 2015; Yap et al. 2005) are appropriate methods to characterize the surface modification as well as undesirable interactions. Differential scanning calorimetry (DSC) is a widely adopted technique for the determination of the glass transition temperature, enthalpy and stability under heating (Argimón et al. 2017; Braithwaite et al. 2017; Jeon et al. 2013). Thermogravimetric analysis (TGA) is another technique generally used for estimation of the amount of vitamin A coupled to the nanocapsule surface, the surface area and encapsulation process (Vilanova and Solans 2015). Ultraviolet spectroscopy and high-performance liquid chromatography are the most widely adopted techniques for determination of vitamin A within nanoparticles.

4.3.3 The Fate of Encapsulated Vitamin a in GIT

Vitamin A nanoparticles are subjected to various unfriendly conditions as it travels inside the GIT which exerts deformation in their structure, composition and flow behaviour. These modifications may manipulate the physiochemical and physiological status of vitamin A, thus influencing its bioavailability. The fate of encapsulated vitamin A in the GIT is watched by those factors which have been intimately coupled with lipid metabolism (triglycerides and phospholipids). These factors involve emulsification, solubilization in micelles, transportation across the stagnant water layer and transmission through enterocyte membranes. The fate of vitamin A in GIT is governed by an array for factors including enzymatic and physiochemical participation. The acidic pH of gastric juice may affect the bioavailability of vitamin A. Further, it is hypothesized that protein digestive enzymes (pepsin and trypsin) may affect the bioavailability of encapsulated vitamin A as they contribute in releasing encapsulated vitamin A from protein-derived nanoparticles. The lipophilic nature of vitamin A encouraged to assume that vitamin A will be more bioavailable if it is incorporated with lipid-derived nanoparticles. This assumption was supported by various studies (Carafa et al. 2008; Chansri et al. 2006; Cristiano et al. 2017; Hwang and Ludescher 2002; Jenning et al. 2000b).

Before absorption of vitamin A by enterocytes, its release from nanoparticles and food matrices and solubilization in GIT fluids is desirable to obtain bioavailability of enterocyte. The lipophilic nature of vitamin A confines its dissolution in aqueous GIT medium. Lipid-derived nanoparticles, such as nanoemulsions, liposome, micelles, and solid lipid nanoparticles, have been widely adopted to improve the bioavailability of lipophilic compounds (Müllertz et al. 2010; Santos and Meireles 2010). In general, digestible carrier oils in nanoparticles are hydrolyzed by lipases

to produce free fatty acids and monoacylglycerols during digestion. These digested fat products interact with bile salts and phospholipids in the GIT to produce "mixed micelles" with complex structures. Vitamin A encapsulated within nanoparticles is transferred to the mixed micelles during the digestion process, which improves its bioaccessibility. The type of carrier oils used in nanoparticle synthesis is key to the bioaccessibility of lipophilic vitamin A. Nanoemulsions comprising primarily of long-chain triglycerides offer greater bioaccessibility of β-carotene than those comprising primarily of medium-chain triglycerides. These data clearly indicate that the nature of carrier oils is crucial to the bioavailability of vitamin A too. Hence, the choice of carrier oils needs to be considered carefully while formulating the lipid-derived nanoparticles for vitamin A in order to enhance its bioavailability. It is also believed that the particle size of nanoparticles may also affect vitamin A bioavailability. This assumption was supported by a focused study in which the nanoemulsions with smaller particles have been exhibited higher bioavailability of β-carotene than those with larger particles (Salvia-Trujillo et al. 2013). Moreover, it was also hypothesized that the nature of surfactants applied in nanoparticle synthesis may influence the bioaccessibility of encapsulated vitamin A. This theory was investigated in simulated study in which it was found that the extent of digestion of carrier triglyceride was positively associated with the hydrophilic/lipophilic balance of the surfactant and negatively associated to the length of aliphatic chain of the surfactant (Speranza et al. 2013). The difference in the oil digestion may exert variation in the solubilization of vitamin A in mixed micelles as a consequence of altered bioavailability. Therefore, the selection of appropriate surfactants is the key to harvest the desired bioavailability.

4.4 Vitamin A Nanoparticles and Food Application

Prevailing vitamin A deficiency and customer awareness are being a major driving force for vitamin A fortification intervention where foods are being used as a platform for vitamin A delivery. This brings various challenges such as solubilization of lipophilic vitamin A, susceptibility against physicochemical stress (pH, oxygen, temperature, UV, pressure), undesirable interaction with food matrices, homogeneity and customer acceptability. Most of the vitamin A fortification interventions adopt direct addition of vitamin A in food matrices followed by homogenization; this brings various limitations such as loss of activity, degradation, heterogeneous distribution, undesirable interaction and alteration in food appearance and taste, hence affecting the customer acceptability. In order to resolve these issues, food technologists adopted various encapsulation techniques for the fortification of target foods. Despite an array of benefits encapsulation techniques remained untapped for food application. The first evidence for the use of encapsulation technique comes from Lui's study where he encapsulated vitamin A in lactoglobulin and applied the encapsulated system in milk fortification (Liu 2003). Further, spray-drying technique was applied for fortifying salt with vitamin A capsule system along with iodine and iron (Zimmermann et al. 2004), Similarly, vitamin A encapsulated in

hydrogenated palm fat and lecithin along with iodine and iron has great potential for food fortification (Wegmüller et al. 2006). Further, liposome-incorporating vitamin A was applied for indirect fortification method to enrich *Artemia nauplii* (fish larvae) (Monroig et al. 2007). Conversely, the high chemical stability of vitamin A in chitosan-derived microcapsule system makes it a very suitable capsule system for food fortification (Albertini et al. 2010). Likewise, Pinkaew and coworkers developed vitamin A premix which have been applied for production of vitamin A-enriched artificial rice (Pinkaew et al. 2012). Similarly, reassembled casein micelles encapsulating vitamin A and D displayed great stability during storage periods which have great potential in milk fortification (Loewen 2014). The potential of the micellar system for encapsulation of vitamin A was further recognized for milk fortification (Mohan 2014). Further, β-lactoglobulin-vitamin A molecular complex has shown its potential in food application (탕가문 2015). N-Vinylcaprolactam, ethylene glycol diacrylate and 2,2′-azobis[2-methylpropionamidine]dihydrochloride-based microgel encapsulating vitamin A displayed great potential for the development of space food (Schroeder 2018).

4.4.1 Safety Concerns and Risks of Vitamin A Nanoparticles

In general, nanoparticles are adapted to improve the oral bioavailability of poorly soluble or labile drugs, or target GIT and lymphatic tissues. The available reports clearly indicate that the uptake of nanoparticles from the GIT is monitored by its particle size (Hillyer and Albrecht 2001) and surface properties (Jani et al. 1989). Similarly modified characteristics of nanoparticles like higher bioavailability, better absorption and controlled release kinetics of vitamin A may transmit undetected risk to the biological system. It is thought that the utilization of biodegradable or natural material may limit health hazards as compared to polymeric nanoparticles. Due to uncertainty in the long or short term and the direct or indirect effect of nanoparticle-derived foods, it is significant to assess the effect of nanoparticles on human health (Dowling 2004). As per food safety, the FDA has planned a special approach coupled with nanoparticle-based food and food components for mass production (Chau et al. 2007). Anyway, there are no definite legislation guidelines framed addressing nanoparticles in the food supply; however, several agencies and government bodies claim to follow the safety concerns of nanoparticle-based food product in their tentative legislative guidelines (Amenta et al. 2015). The guidelines have made a list of suggestions: (i) the physiochemical characterization of nanoparticles applied in food; (ii) characterization process to assess their hazard characteristics embraced by nanoparticles, such as long- and short-term toxicity assay; (iii) submission of a toxicity assessment report to legislative bodies such as the FDA, EU and FSSAI; (iv) recognizing and stating a regulatory compliance for the consumption of the nanoparticle-derived foods. However, the lack of guidelines regarding nanoparticle-derived foods demands various legislative bodies to come together to frame universal guidelines for nanomaterial-derived food products which can be applied across the globe.

4.5 Conclusions

Encapsulation seems to be an indispensable means to improve the bioavailability of vitamin A. Accruing reports have highlighted that nanoparticles can be applied to improve their potential health benefits in humans to combat the associated disorders. It is noticeable that vitamin A displays greater bioavailability when it is incorporated in lipid-derived nanoparticles as compared to polymer-based nanoparticles. Additionally, it was also observed that the quantity, degree of saturation of carrier oil, nature of surfactant and adopted encapsulation technique are the key to the defined bioavailability. More systematic mechanistic approaches are needed to carve the correlation between the nanoparticle attributes and their effect on the biological fate of incorporated lipophilic vitamin A. Update in vitamin A encapsulation may offer solid scientific information for the rational designing of novel nanoparticles incorporated to improve the bioavailability of vitamin A which could be applied to other vitamins as well as lipophilic bioactive compounds.

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Chapter 5
Antimicrobial Activity of Nanomaterials

Bablu Lal Rajak, Rahul Kumar, Manashjit Gogoi, and Sanjukta Patra

Contents

Abstract The World Health Organization reports that millions of deaths occurring worldwide are because of infectious diseases caused by bacteria, viruses, fungi and parasites. The existing therapeutics is not adequate enough to fight against these diseases and their prolonged uses have led to the development of drug-resistant strains which are even more difficult to control. Hence, the need for an alternative approach is growing. Development of nanotechnology, especially nanostructured particles and formulations, is providing new opportunities to combat these infectious diseases more effectively. Nanomaterials have unique physicochemical

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properties like tuneable size, large surface to volume ratio, high reactivity, biocompatibility and functionalizable surface area. These properties are applied to facilitate the applications of antimicrobial drugs, thereby overcoming some of the limitations of traditional antimicrobial therapeutics. Moreover, the therapeutic effect and drug delivery approach of these nanomaterials have emerged as an innovative and promising alternative that enhance therapeutic effectiveness against pathogenic microorganisms and minimize undesirable side effects of the drugs. In order to enumerate the antimicrobial effect of these nanomaterials, this chapter is designed to discuss commonly used nanomaterials such as lipid vesicle dendrimers, polymeric and inorganic nanoparticles, carbon nanostructures, quantum dots, electrospun nanofibres, nanoclays, etc. against infectious diseases.

Keywords Antimicrobial · Dendrimers · Lipid vesicles · Nanoclays · Nanofibers · Quantum dots

5.1 Introduction

Microorganisms, as the name suggest, are microscopic living organisms that are visible with the help of aided microscopic devices. They have inhabited on earth for more than 3.5 billion years and are regarded as the first form of life on the planet. Most of these microorganisms are unicellular (single-celled) such as bacteria but few are multicellular such as algae and fungi. They survive in different environments and their habitat ranges from ice cold climate to hot springs, deserts to marshy lands and skin surfaces to the gut. Though they are omnipresent, their presence in the environment may be beneficial or harmful to others. The association of useful microorganisms such as bacteria and fungi with humans is as old as the civilization. Their important role in different nutrient cycles, decomposition of harmful chemical pollutants and wastes, fermentation, digestion of food and protection from harmful microbes in the body, production of vaccines and antibiotics, genetic engineering and biotechnology is effectively utilized in different applications for the benefit of humans (Tortora et al. 2004). Similar is the case with pathogenic (harmful) microorganisms that cause infections and diseases such as dysentery, diarrhoea, tuberculosis and cholera in humans. These pathogenic microorganisms have received significant attention due to their harmful effects leading to suffering and death in humans. In 2015, the World Health Organization (WHO) estimated that 3.2 million deaths worldwide were due to respiratory infections and 1.4 million deaths due to diarrhoeal diseases and tuberculosis each (WHO 2015). The report briefly showed the magnitude of threat these pathogenic microbes are causing to the human population and how important it is to control their growth through therapeutic approaches. Moreover, the emergence of antimicrobial resistance (AMR) strains of bacteria, fungi and parasites is becoming a serious threat to public health leading to disease severity and their treatment (Roca et al. 2015). Globally, it is found that around

700,000 deaths occur each year due to resistance to antimicrobial drugs by emerging strains of mutant microorganisms. It is estimated that such AMR strains of organisms would be accountable for the death of around 10 million people worldwide by 2050 (Robinson et al. 2016). In order to conquer deaths caused by infectious diseases and avoid the emergence of any resistant strains, researchers worldwide are looking for alternatives that can be used against a broad range of microbial populations. New alternatives to antibiotics have been identified till date including antibodies, probiotics, bacteriophages, vaccines and antibiofilm peptides that can be used against infectious diseases (Czaplewski et al. 2016; François et al. 2016; Ploegmakers et al. 2017; Wang et al. 2016). In addition to these, various nanostructures and nanoformulations with existing drugs were found to be effective against different infectious diseases (Malmsten 2014; Karaman et al. 2017; Raghunath and Perumal 2017). These nanostructures interact physiochemically with the cells and cellular organelles for effective therapeutic treatment (Nel et al. 2009). These physiochemical interactions lead to reorientation of the metabolic pathways inside the cells disturbing the biological mechanisms like protein folding, membrane dynamics, enzyme catalysis and DNA replication. which inhibit microbial growth (Moyano and Rotello 2011; Dewan et al. 2014). Additionally, the generation of reactive oxygen species (ROS), metal-ion release, nanoparticle internalization into cells and direct mechanical destruction of the cell wall and/or membrane by the nanomaterials contribute to the disruption/deaths of microorganisms (Pelgrift and Friedman 2013). Irrespective of the mechanism of microbial cell death, nanomaterials are giving hopes for an alternative to age-old therapeutic agents used till date. The use of different nanostructures such as liposomes, dendrimers, quantum dots, nanoclays and other nanoparticles serves a dual purpose against infectious diseases: firstly, they themselves possess therapeutic properties that inhibit the proliferation of microbial growth and secondly, they aid drug delivery by transporting drugs to the target site of action which otherwise was not possible directly. In this chapter, the therapeutic potential of nanomaterials such as lipid vesicles, dendrimers, polymeric and inorganic nanoparticles, nanofibres, nanoclays, quantum dots and carbon nanomaterials is discussed along with brief description of the diseases caused by microbes such as bacteria, fungi, protozoa and viruses and their existing therapeutics.

5.2 Microbial Diseases and Their Existing Therapeutics

Most people link microorganisms as disease-causing agents, but not all microorganisms are harmful (Tortora et al. 2004). The beneficial processes of microbes include decomposition of dead plants and animals; protection against harmful pathogens by altering the pH, acidity level, releasing toxins and regulating and stimulating the immune system (Calder and Field 2002; Reid and Burton 2002). On the contrary,

harmful microbes cause diseases in humans by defeating the immune system and eliciting their harmful effect. The mechanisms followed by these microorganisms to cause illness in humans are either through rapid multiplication inside the host that disrupts the normal function of the organs or destruction of metabolic machinery of the cells/tissues by the production of toxins (Fauci 2004). Several microorganisms responsible for causing diseases in humans are species of bacteria, fungi, protozoa and viruses that enter the body by contact (infected skin, mucous membranes and body fluids), contaminated food and water, blood and vectors such as fleas, mites, ticks and mosquitoes. Common diseases such as pneumonia, bronchitis, whooping cough and tuberculosis (affecting the respiratory tract); typhoid fever, cholera, botulism, peptic ulcer, dysentery and food poisoning (affecting gastrointestinal tract); urinary tract infections; and skin infections are mostly caused by bacterial species of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Haemophilus*, *Enterobacter*, *Mycobacterium*, etc. Moreover, diseases such as aspergillosis, candidiasis, ringworm and some skin infections are caused by fungi species, namely, *Aspergillus, Candida, Tinea and Cryptococcus*, whereas malaria is caused by a protozoon, *Plasmodium*. However, infections like common cold, influenza, meningitis, encephalitis, chikungunya, chicken pox and AIDS are caused by viruses (Goering et al. 2018).

In order to combat any infection, the defence mechanism of our body is immediately elicited. It is well known that the T-cells are responsible for antimicrobial activity by producing lymphokines at the site of infection (Reinhardt et al. 2001). Failure of this internal defence system against microorganisms leads to infection, and then therapeutic treatment is required. Conventionally, the use of plant extracts, aromatic herbs, essential oils, etc. occurring naturally had been in use as antimicrobial agents to treat a number of infectious diseases around the world, but the discovery of antibiotics leads to a new therapeutic treatment approach (Khan et al. 2009; Solórzano-Santos and Miranda-Novales, 2012). Antibiotics are metabolites produced by certain microorganisms naturally or their semisynthetic derivatives, which inhibit the growth of certain other microorganisms. The first discovered antibiotic penicillin produced by a fungus *Penicillium chrysogenum* was extensively used during World War II to control the spread of infectious diseases. Since then, several other antibiotics, namely, actinomycin, erythromycin, rifamycin, streptomycin, tetracycline and vancomycin produced by species *Streptomyces*; bacitracin and polymyxin by *Bacillus*; and cephalosporin by *Cephalosporium*, are till date being used for the treatment of different infections caused by bacteria (Finch et al. 2010). In cases of fungal infections, the treatment regimen is often difficult to formulate because human cells, also being eukaryotic are susceptible to harm. In order to circumvent this, antibiotics such as amphotericin B; nystatin; griseofulvin in combination with synthetic imidazoles, triazoles and their derivatives; and pyrimidine analogues are commonly used (Denning and Hope 2010). However, antiviral drugs such as acetaminophen and ibuprofen against common cold and flu; acyclovir, valaciclovir, etc. against herpes virus; human recombinant interferon alpha and PEGylated interferon alpha against hepatitis B; and zidovudine, didanosine, tenofovir disoproxil, etc. against HIV hinder the ability of these viruses to reproduce and

control their spread (De Clercq 2004). In addition to these, combined drug therapies are used to treat diseases caused by protozoa, for example, metronidazole and iodoquinol against amoebiasis; amphotericin B and chlorpromazine against amoebic meningoencephalitis; artemisinin and metal-based therapy against malaria, trypanosomiasis and leishmaniasis (Sayang et al. 2009; Navarro et al. 2010).

Although good medical progress was made during the last century in developing antibiotics and chemically derived synthetic analogues, infections still remain a major public health problem worldwide. This problem is further aggravated by the emergence of antimicrobial resistance (AMR) strains that occurred due to prolonged exposure to similar drugs, administered in different ways and diseases worldwide. Though the mechanism of development of AMR is not fully understood, several mechanisms have been described, including the acquisition of antibiotic resistance genes via the transfer of genetic elements or mutations leading to altered expression of redox-active proteins, altered drug metabolism either by substitution or degradation, changing the chemical composition of cell wall leading to decreased permeability of drugs, etc. (Yelin and Kishony 2018), as well as the formation of biofilms (Peng et al. 2017). The well-known mechanism of development of AMR strains (schematically represented in Fig. 5.1) includes: (i) the formation of modified cell walls that restrict the penetration of drugs into the cell, (ii) production of chemically active molecules that conjugate with the drug molecules and render them inactive, (iii) increased channel activity that pumps out the drug molecules and (iv) production of modified binding receptors that are unable to bind to the drug molecules.

The emergence of AMR strains of microorganisms is becoming a serious threat to human population in the twenty-first century which demands for a new treatment regimen so that millions of deaths can be avoided in the future. This is possible only through the synthesis or discovery of active novel molecules and their encapsulation within nanomaterials so that the drug can reach the cellular organelles where pathogens reside and kill the pathogens without harming the patients (Ogawa et al. 2018).

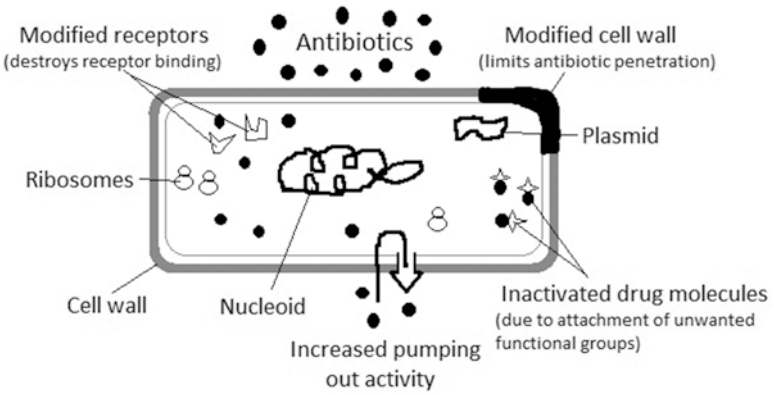

Fig. 5.1 Mechanism of antimicrobial resistance. (Adopted from Singh et al. 2014)

5.3 Nanostructured Materials as Antimicrobial Agents

Nanostructured materials are seen as medical alternatives to antibiotics due to the capability to tailor them for specific diseases and site-specific targeted delivery. It is obvious that for pharmaceutical agents to render their therapeutic effect, the primary targets must be within cells and tissues so that selective subcellular delivery is likely to have greater benefit. Several organic and inorganic nanomaterials are currently in clinical and preclinical stages that have potential therapeutic effects. The nanomaterials with their noble properties such as size, surface to volume ratio, reactivity, biocompatibility and tunability offer biologically active domain for site-specific targeting, drug delivery, biocompatible coatings, etc. which can be engineered for healthcare applications (Fig. 5.2). Most engineered nanomaterials acting as drug delivery system and as therapeutic agents against infectious diseases are liposomes, dendrimers, polymeric nanoparticles, carbon nanostructures, quantum dots, electrospun nanofibres, nanoribbons, core-shell nanoparticles, etc. and are discussed in the following sections.

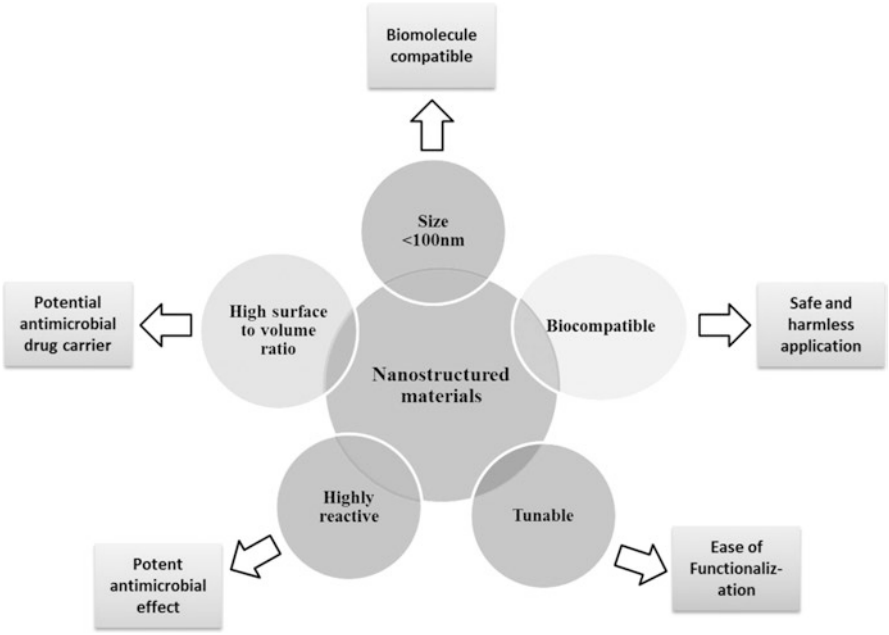

Fig. 5.2 Properties of nanostructured materials that make it potent antimicrobial agents

5.3.1 *Lipid Vesicles*

Lipid vesicles are composed of either mono- or bilayers of phospholipids with size ranging between 50 and 100 nm. The bilayer structures of phospholipids are known as liposomes and the monolayered ones are called micelles, whereas solid lipid nanoparticles (SLNs) are composed of a solid lipid core encapsulated with drugs and the nanocapsules consist of a liquid core with shell-type surface (Fig. 5.3).

The structural morphology of these lipid vesicles enables them to encapsulate a wide variety of hydrophilic and hydrophobic diagnostic or therapeutic agents, providing a good drug payload per particle and protecting the encapsulated drugs from metabolic processes. It is important to note that drug entrapped in these vesicles is bioavailable with or without stimulus such as pH and temperature. Moreover, the ability of accumulated lipid vesicles to increase the local bioavailable drug concentrations and their therapeutic outcome can only be enhanced when the rate of release of entrapped drug from these nanostructures is optimized (Johnston et al. 2006).

Conventional vesicles suffered drawbacks because of their rapid degradation following plasma protein adsorption. The next generation of these vesicles were designed to overcome this drawback by coating the surface with polymer derivatives such as polyethylene glycol (PEG) or carbohydrates. These sterically stable nanostructures have been shown to favourably work as drug delivery vehicles that withstand the metabolic processes and perform drug release in a controlled manner (Torchilin 2005). The mechanism of drug delivery using these lipid vesicles into the cell is performed in stages (Fig. 5.4); in the first stage, the nanovesicle-cell interaction occurs where they nonspecifically or specifically bind to the cell surface. Nonspecific adsorption occurs by simply an electrostatic and/or hydrophobic interaction between the two, while specific adsorption is a receptor-ligand or an antigen-antibody interaction between the two surfaces of the cell and the nanovesicle. Irrespective of whether the binding is specific or nonspecific, the nanovesicle is internalized into the cell by endocytosis. This is followed by the enzymatic digestion of the liposome in the intracellular compartment such as endosome, phagosome or acidosome, accompanied by the intracellular distribution of drugs to the cytosol (Daraee et al. 2016).

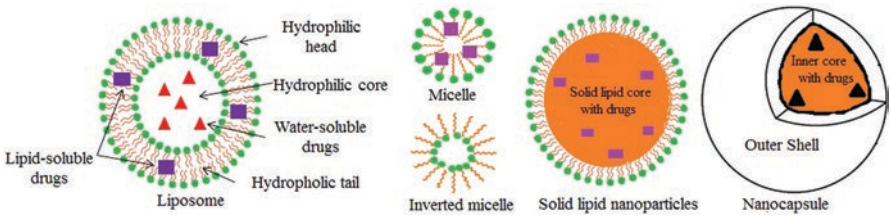

Fig. 5.3 Structure of lipid vesicles such as liposomes, micelle, solid lipid nanoparticles and nanocapsules containing entrapped drugs

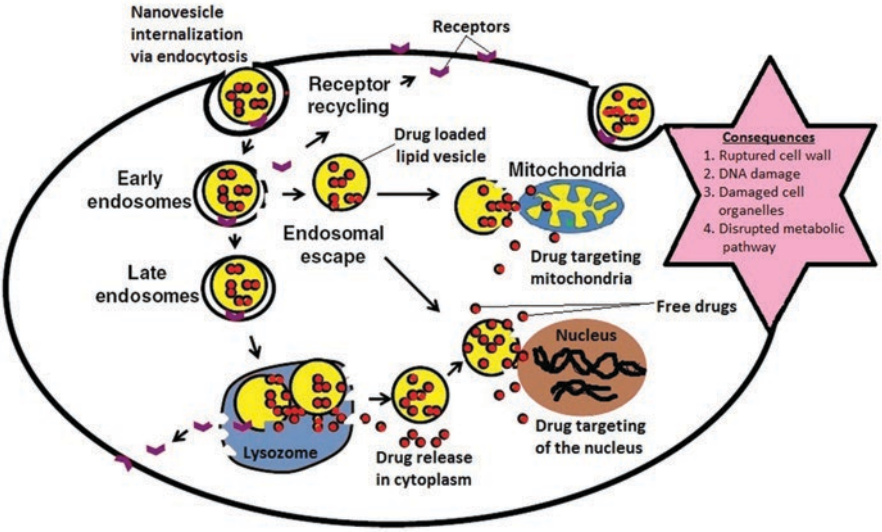

Fig. 5.4 Mechanism of drug delivery using nanovesicles with possible causes of microbial cell death. (Adopted from Çağdaş et al. 2014)

Liposomes were the first vesicular structure to be explored by encapsulating antibiotics and bioactive molecules to increase the therapeutic dose of the formulation, circulation time and bioavailability as compared to the free drug (Pinto-Alphandary et al. 2000; Barratt 2003). Mikasome, an amikacin liposomal formulation, was found to be more potent than the free drug against murine tuberculosis (Donald et al. 2001). Similarly, pulmonary administration of solid lipid nanoparticles containing rifabutin was reported to enhance antibacterial activity of *Mycobacterium tuberculosis* in a murine model (Gaspar et al. 2017). Improved bioavailability of kaempferol, a flavonoid compound, was achieved when loaded into lecithin/chitosan nanoparticles that proved to be potent against a pathogenic fungus *Fusarium oxysporum* (Ilk et al. 2017). Additionally, liposomes loaded with antibiotics have demonstrated excellent transportation capability and severalfold increase in potency in both in vitro and in vivo studies against *Pseudomonas, Salmonella*, *Streptococcus* and others (Pushparaj Selvadoss et al. 2018; Lakshminarayanan et al. 2018). Similar drug transportation potential was also seen in other lipid-based vesicular structures; i.e., dehydroascorbic acid (DHA)-coupled polymeric nanomicelles encapsulating itraconazole were effectively transported across the blood-brain barrier that showed high efficacy in a murine model of *Cryptococcus neoformans* infection of the central nervous system (Shao et al. 2015). The enrofloxacin-loaded docosanoic acid solid lipid nanoparticles with different physicochemical properties were developed to enhance intracellular activity against *Salmonella* and were considered to be a promising drug carrier (Xie et al. 2017). The antibiofilm activity of liposomal levofloxacin and lysozyme improved severalfold against lung infection caused by *S. aureus* in rats (Gupta et al. 2018). Additionally, lipid nanocapsule loaded with

antipsychotic agents such as chlorpromazine and thioridazine improved its overall uptake in bacteria and effectively inhibited proliferation of gram-positive *S. aureus* and gram-negative *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* bacteria *in vitro* (Nehme et al. 2018).

Liposomes loaded with bioactive lipids, cinnamon oil, chitosan, peptides, etc. have been found to be effective in different strains of bacterial populations along with those of resistant strains (Cui et al. 2016; Poerio et al. 2017; Pu and Tang, 2017). Essential oils, such as eucalyptus or rosemary oils, loaded with solid lipid nanoparticles were able to promote wound healing in rats and found to be effective against *S. aureus* and *Streptococcus pyogenes* (Saporito et al. 2018). Moreover, antimicrobial suspension of triclosan and α-bisabolol encapsulated in chitosan-coated nanocapsule inhibiting a pathogenic strain of *P. aeruginosa* resistant to triclosan became susceptible to a dose nearly eightfold smaller and was thus used commonly in wound dressing (Marchi et al. 2017).

Furthermore, liposomal formulations seemed superior for the treatment of fungal and parasitic diseases compared to their free drug counterpart. In many examples, the toxicity of the antibiotic was dramatically reduced which enable larger amounts of drug targeting to the infected tissues. This increased the efficacy of the treatment by increasing the therapeutic index of liposomal formulation and reducing the side effects. An excellent example to compliment the above statement is the liposomal formulation of amphotericin B, which is the leading drug against leishmaniasis and other fungal infections. The liposome encapsulation reduced its toxicity by 50–70-fold, which allowed more than fivefold administration as compared to conventional treatment. The nanoliposome formulations such as AmBisome® and DepoCyt[e] are today marketed as the most effective treatment for leishmaniasis and other fungal infections which are FDA approved (Sundar and Prajapati 2012). Besides AmBisome®, other formulations of amphotericin B lipid nanostructures were reported to be effective in amoebic meningitis, candidiasis and invasive fungal infections, even in immune-compromised patients (Ringden et al. 1991; Cornely et al. 2007). Nanomicelles of amphotericin B and sodium deoxycholate sulphate when used as aerosol inhalation for lung infection were reported to inhibit *Cryptococcus neoformans* and *Candida albicans* and were also found to significantly improve antileishmanial activity (Usman et al. 2018). Another liposomal formulation under investigation is buparvaquone that has an immunomodulatory effect on the host cells and is highly effective at low doses in eliminating *Leishmania infantum* parasites (da Costa-Silva et al. 2017).

Several other liposomal formulations have also been reported as effective antiviral agents; for example, polyunsaturated endoplasmic reticulum liposomes, commonly known as PERL, target the cholesterol synthesis within infected cells in a large number of viral systems, including hepatitis C virus (HCV), hepatitis B virus (HBV) and HIV (Pollock et al. 2010). The matrix 2 protein ectodomain segments (M2eA) corresponding to the H1N1, H5N1 and H9N2 influenza strains were formulated using a novel liposome-based vaccine technology and were evaluated as potential immunogens which could be used for the development of influenza vaccine (Ernst et al. 2006). At the moment, a number of liposome formulations are in

clinical trials as an adjuvant for prophylactic as well as therapeutic vaccines against malaria, influenza, tuberculosis (TB), human immunodeficiency virus (HIV) and dengue fever, whereas Cervarix®, Inflexal®, Epaxal® and Gardasil® are commercially available liposome vaccines against infection by human papilloma virus (HPV), influenza virus and hepatitis A virus, respectively (Bernasconi et al. 2016). Polymeric nanocapsules consisting of protamine and arginine-rich polymers were recently reported to elicit higher protective immune response as recombinant hepatitis B surface antigen in mice model which may become an alternative antigen delivery vehicle (Peleteiro Olmedo et al. 2018).

5.3.2 Dendrimers

Dendrimers are hyperbranched monodispersed macromolecules with low polydispersity with micelle-like behaviour and nano-reservoir properties (Fig. 5.5a). Dendrimer is a three-dimensional globular structure consisting of a central core, an interior dendritic structure (the branches) and an exterior surface with functional groups, all made up of polymers (Svenson and Tomalia 2012). They differ from classical polymers in two main characteristics: firstly, they are never synthesized by polymerization reactions, instead a step-by-step process, affording to a perfectly defined and highly reproducible structure, and secondly, they have a highly branched

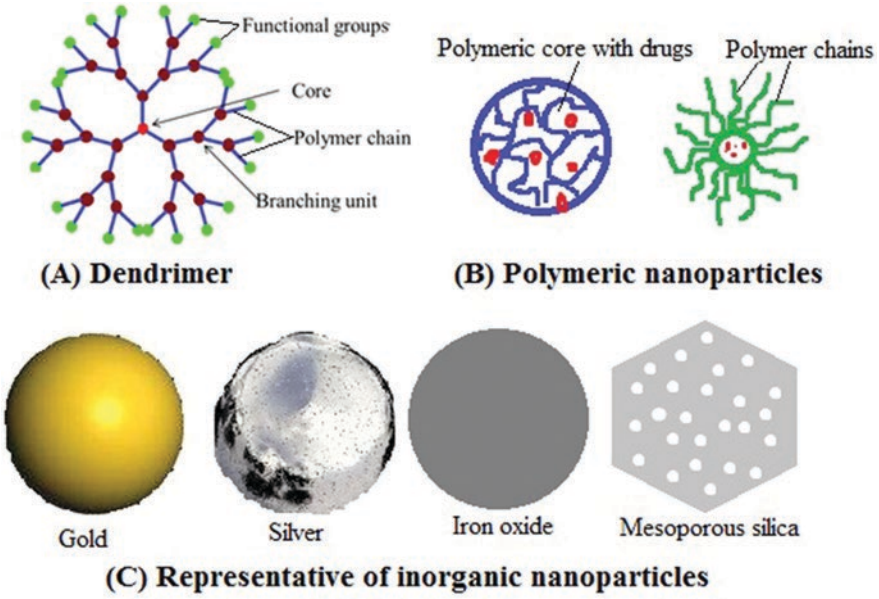

Fig. 5.5 Structures of (**a**) dendrimer with a core and polymer chain, (**b**) polymeric nanoparticles with hydrophilic and hydrophobic core and (**c**) inorganic nanoparticles

3D architecture due to the use of at least one type of branching units as building blocks for their synthesis. Their peculiar structure, reasonable cost of manufacture, toxicological profile and biocompatibility distinguished them from other nanosized species used for polyvalent or multivalent drug discovery/delivery.

Most commonly used polymers for the synthesis of dendrimers are polyamidoamine (PAMAM) and polypropylene imine (PPI). Both PAMAN- and PPI-derived dendrimers have been reported to possess therapeutic value in treating viral and bacterial diseases as well as inflammation (Gong et al. 2002; Chauhan and Jain, 2003). Though dendrimers are known to possess therapeutic properties, they are highly toxic. In order to reduce their cytotoxicity, they are often modified with PEG, carbohydrates, hydroxyl or carboxyl groups to improve their surface activities, as well as their biological and physical properties (Gajbhiye et al. 2007; Ziemba et al. 2011; Kolhatkar et al. 2007).

The antimicrobial potential of dendrimers depends largely upon the type and size of the attached functional groups. Smaller dendrimers are effective, as bulkier dendrimers are unable to pass through the cell membrane and have difficulty in reaching the target site for the anticipated antimicrobial action (Sadegh-Hassani and Nafchi 2014). Amino-terminated PAMAM was found to possess strong antibacterial activity as compared to hydroxyl-PAMAM and carboxyl-PAMAM. This is because the protonated amino group on PAMAM promotes the disruption of the bacterial membrane through electrostatic interaction (Xue et al. 2015). Thus, antimicrobial activity of dendrimers is mostly due to their cationic interaction with the negatively charged bacterial cells. These interactions increase internalization of dendrimers and destroy the membrane proteins which disturb the potassium ion distribution around the bacterial cells. The disturbance caused by the dendrimers completely disintegrates the bacterial membrane causing a bactericidal effect (Chen and Cooper 2002; Cheng et al. 2007). Biocompatible phloroglucinol succinic acid dendrimers were reported to possess an inhibitory effect against a number of gram-positive and gram-negative bacteria (Kumar et al. 2015). Another class of amine- and ammonium-terminated carbosilane cationic dendrimers has demonstrated antimicrobial activity against both gram-positive and gram-negative bacteria (Ortega et al. 2008). Carbosilane dendrimers and dendrons functionalized with guanidine were found to be microbicidal against *E. coli*, *Staphylococcus aureus* and methicillin-resistant *S. aureus* bacteria and against *Acanthamoeba polyphaga* (Heredero-Bermejo et al. 2018). Additionally, hyperbranched PAMAM functionalized with N-diazeniumdiolate nitric oxide, a nitrous oxide (NO) donor, proved effective against common dental pathogens (Yang et al. 2018). Moreover, the conjugated polyglycerols with O-carboxymethylated chitosan and boron suppressed the proliferation of *S. aureus* and *Pseudomonas aeruginosa* (de Queiroz et al. 2006). Additionally, the poly(quaternary ammonium) polymers were engineered for antibacterial specificity and their ability to delay the development of bacterial resistance. These linear poly(quaternary ammonium) homopolymers and block copolymers showed structure-dependent antibacterial specificity toward gram-positive and gram-negative bacterial species by mimicking the behaviour of surface-presented polycationic biocides (Ji et al. 2017).

As an antifungal agent, PPI was shown to improve the solubility of clotrimazole and enhance its antifungal activity against species of *Candida* (Winnicka et al. 2011). Dendrimeric lipopeptides were reported to cause morphological changes in fungal cells and inhibit the enzyme activity of 1,3-β-d-glucan synthase in *Candida* (Janiszewska et al. 2012). The development of dendrimeric peptides (multiple strand protein conjugates) with lysine core was also found to be potent against a number of bacterial species (Tam et al. 2002; Scorciapino et al. 2012) and efficiently kill gram-negative bacteria including the two of the most problematic multidrug-resistant bacteria worldwide *P. aeruginosa* and *Acinetobacter baumannii* (Siriwardena et al. 2017). The central role of peptides in eliciting immune response and development of vaccines against infectious diseases including viral diseases are emerging which can be the most cost-effective methods of improving public health. Induction of immune responses by DNA vaccines formulated with dendrimer and poly-methyl methacrylate (PMMA) was strong and effective in inducing specific antibody and cellular responses thereby reducing the parasite *Leishmania* in mice model (Tabatabaie et al. 2018). Additionally, the DNA vaccines based upon PAMAM-lysine elicited a predominant antibody response with an increase in the production of interleukins (IL-2) to provide protection against *Schistosomiasis japonica* infection (Wang et al. 2014).

5.3.3 Polymeric Nanoparticles

Polymeric nanoparticles (PNPs) are one of the most studied organic nanostructures for application in nanomedicine because it is prepared from either natural or semi-synthetic polymers. Due to their synthetic precursors, they can entrap drug molecules in its lipid core or may be covalently bonded to the drugs (Fig. 5.5b). These PNPs are stable, biodegradable and biocompatible and can be easily distributed in the living system due to their building block similarity with biological components. The drug or bioactive molecules in PNP are either dissolved or entrapped or encapsulated or attached to a nanoparticle matrix which can thus improve the diagnosis and treatment of a wide range of diseases, ranging from cancer, viral infections and cardiovascular diseases to pulmonary and urinary tract infections (Hajipour et al. 2012). In the polymeric antimicrobial drug delivery systems, drug molecules can be incorporated in the core of the particles or covalently or non-covalently bonded on the surface of polymeric nanocarriers or encapsulated in the PNPs (Michalak et al. 2016).

Another group of PNPs include nanohydrogels which are extraordinary nanostructures that have the capability to hold a large quantity of water within them. These substances with high water content are synthesized from cross-linked polymers that also have the ability to deliver various drugs or a variety of therapeutic agents in the living system. The first well-known hydrogel developed for biomedical applications was polyhydroxyethyl methacrylate that enabled self-regulated drug delivery systems (Lee et al. 2013). The polymer-based nanoparticles' applica-

tions include drug delivery, wound healing (Greenhalgh and Turos 2009) and antimicrobial activity (Torus et al. 2007). These nanostructures being synthesized using non-biodegradable polymers, such as poly(methyl methacrylate) (PMMA), polyacrylamide, polystyrene and polyacrylates (Torus et al. 2007; Bettencourt and Almeida 2012; Vijayan et al. 2013) suffer from their disadvantageous traits such as chronic toxicity and inflammatory reactions, leading to a shift towards biodegradable polymers. Biodegradable polymers include synthetic polymers such as poly(lactide) (PLA), poly(lactide-co-glycolide) copolymers (PLGA), poly(ε-caprolactone) (PCL) and poly(amino acids) in addition to natural polymers such as chitosan, alginate, gelatin and albumin (Elsabahy and Wooley 2012; Zhang et al. 2013).

Generally, PNPs may interact with the bacterial cell wall either via passive or active targeting. Passive targeting is based on particle size and the ability of particles to disturb the cell wall of bacterial membrane and damaging it. For active targeting of PNPs, the surface of polymeric nanoparticles is usually functionalized with specific antibodies and aptamer bacteriophage proteins that provide specific identification of the pathogens and interaction between the particles and pathogens. The reported studies revealed that both the active and passive targeting strategies to deliver antimicrobial agents with PNPs improve their activities compared to their free form (Kavruk et al. 2015; Barreras et al. 2016). To date, a significant number of reports on the activity of antibiotic-conjugated polymeric nanoparticles against various infections, including those caused by drug-resistant pathogens, have been published. The most common is chitosan nanoparticle either alone or loaded with different metal ions such as copper, manganese, zinc, iron and silver that caused an inhibitory effect in numerous gram-positive and gram-negative bacteria including multidrug-resistant strains (Qi et al. 2004; Du et al. 2009; de Paz et al. 2011; Cremar et al. 2018). The cationic chitosan nanoparticles interact with the anionic surfaces of the microbial cell membrane thereby hindering microbial activity. Chitosan nanoparticle being a biocompatible antioxidant possesses an inhibitory effect against *Candida albicans* (Mubarak Ali et al. 2018) and *Fusarium oxysporum* (Dananjaya et al. 2017). However, in pulmonary infection associated with *P. aeruginosa*, tobramycin alginate/chitosan nanoparticles demonstrated DNA degradation and improved nanoparticle penetration (Deacon et al. 2015). A similar effect was reported using nanohydrogels, for example, ZnO nanoparticles incorporated in nanohydrogel particles made out of sodium alginate/gum acacia and cross-linker glutaraldehyde ensured their gradual and sustained release and demonstrated desired level of antibiotic activity against *P. aeruginosa* (Chopra et al. 2015). Moreover, delivery of levofloxacin, a fluoroquinolone antibiotic scarcely efficient in intracellular infections, entrapped within polysaccharide nanohydrogels efficiently increased the antibacterial activity of the formulation against *P. aeruginosa* and *S. aureus* (Montanari et al. 2014). However, biocompatible PNPs composed of chitosan/sodium tripolyphosphate (TPP) and encapsulated with mercaptosuccinic acid (MSA) acted as spontaneous nitric oxide (NO) donors, with free NO release showing a significant decrease in the percentage of macrophage infected with amastigotes of *Trypanosoma cruzi* (Seabra et al. 2015).

Furthermore, antibacterial property of PMMA containing silver nanofibre was reported against *E. coli* and *S. aureus*, where release of biocidal Ag^+ ions from polymer matrix embedded with silver bromide nanoparticles was able to kill both airborne and waterborne bacteria and also resisted the formation of biofilms (Kong and Jang 2008; Sambhy et al. 2006). Furthermore, drug-loaded PNPs offer added advantages with the ability of stimuli-responsive release of drugs, for example, levofloxacin-loaded PNPs and ciprofloxacin-loaded PNPs against biofilm cells of *E. coli* (Cheow et al. 2010; Singh et al. 2018). Another drug-encapsulated, pH-responsive, surface charge-switching poly(d,l-lactic-co-glycolic acid)-b-poly(l-histidine)-b-poly(ethylene glycol) nanoparticles were able to potentially treat gram-positive, gram-negative and polymicrobial infections associated with acidity (Radovic-Moreno et al. 2012). Similarly, nystatin-loaded PLGA and PLGA-glucosamine nanoparticles exhibited higher antifungal activity (Mohammadi et al. 2017).

5.3.4 Inorganic Nanoparticles

Inorganic nanoparticles, including gold, silver and oxides of iron, titanium, zinc or silicon, and ceramic nanoparticles such as silica and alumina are continuously being investigated in both preclinical and clinical studies for the treatment, diagnosis and detection of many diseases (McCarthy and Weissleder 2008; Na et al. 2009; Giljohann et al. 2010; Huang et al. 2011; Li et al. 2012). Many inorganic metals such as platinum (e.g. cisplatin, carboplatin, oxaliplatin), gold, silver and copper had been in clinical use for centuries, but the understanding of their antimicrobial effect is only a few decades old due to recent studies in their nanoscale dimensions (Zhang and Lippard 2003; Harper et al. 2010). The significant changes in the property of materials that exist in their nanoscale dimension compared to their bulk counterparts are the only reason for their exploration in the field of nanomedicine. It is established that as the size of the material decreases, the proportion of surface atoms increases, thereby increasing the reactivity of these surface atoms (Hanemann and Szabó, 2010). Inorganic nanoparticles are currently explored for their potential use both as therapeutics and drug delivery agents because of the advantage of chemical and mechanical stability as well as surface functionalization with tunable particle size and morphology. Another reason for which inorganic nanoparticles have emerged as potential antimicrobial agents is their relatively low cost, low toxicity and biocompatibility (Huh and Kwon 2011). Silver nanoparticles are known to possess antibacterial and antiviral properties that even acts against HIV and hepatitis viruses (Galdiero et al. 2011). Similar is the case with multivalent gold nanoparticles (Bowman et al. 2008). Recently, nanostructured oxides consisting of two or more metallic components forming core-shell architecture such as Ag-SiO2, Fe3O4/TiO2 and Ag/Fe3O4 demonstrated promising results due to their unique physiochemical properties (Cioffi et al. 2005; Chen et al. 2008; Banerjee et al. 2011). The monometallic gold and silver and bimetallic gold-silver nanoparticles with biologi-

cal activity against five opportunistic *Candida* strains demonstrated high antifungal activity against *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. guilliermondii* and *C. albicans* (Gutiérrez et al. 2018). In malaria, metal-chelating agents seem to be promising therapeutic adjuvants for treatment against severe *Plasmodium falciparum* infection, and ferroquine, an iron-chloroquine derivative, has been found active against both chloroquine-susceptible and chloroquine-resistant *P. falciparum* and *P. vivax* strains (Sekhon and Bimal 2012).

In general, the inorganic nanoparticles may be engineered to evade the pathogenic system by varying their size and composition (Fig. 5.5c). They may be porous and act as a reservoir to physically encage and protect an entrapped molecular payload from degradation or denaturisation, or may allow surface interaction to hold the drug molecule just as ligand binding (Roy et al. 2003). Like their organic therapeutic counterparts, inorganic therapeutics can benefit from being formulated as a nanoparticle delivery system to improve their biological performance by enhancing pathological targeting, drug loading and immune system evasion (Farokhzad and Langer 2009; Peer et al. 2007). Certain inorganic nanoparticles can respond to specific external stimuli such as magnetic fields or near-infrared light to facilitate on-demand drug release (Timko et al. 2014). The advantage of using these inorganic nanomaterials as antimicrobial agents is that they contain mineral elements essential to humans and exhibit strong activity even when administered in small amounts. Inorganic nanoparticles are particularly interesting because they can be prepared with tuneable morphology. It has already been established that the antibacterial activity of inorganic nanostructures is directly influenced by different structural morphologies (Zhang et al. 2007; Talebian et al. 2013).

Several metal (Au and Ag) and metal oxide (ZnO, CuO, NiO, Sb_2O_3, MgO, Gd_2O_3, SnO_2, WO_3, ZrO_2, Fe_2O_3, TiO_2, CeO_2, Al_2O_3, Bi_2O_3, etc.) nanoparticles have been shown to inhibit the growth of different gram-positive and gram-negative bacteria by changing the membrane permeability, altering metabolic pathways, affecting DNA replication followed by altering transcription and translation processes and most importantly by increasing the intracellular level of metal ions (Applerot et al. 2012; Zhou et al. 2012, Horie et al. 2012). Though the exact mechanism of antimicrobial activity caused by these metallic nanoparticles is not completely understood, there are strong evidences that the inhibition is caused by the generation of reactive oxygen species (like hydroxyl radicals or superoxide anions or hydrogen peroxide), or oxidative stress or free metal ion toxicity arising from the dissolution of metals from the surface of the nanoparticles or the combination of one or more processes that disrupts the normal metabolic activities of the organism thereby killing them. Furthermore, morphological and physicochemical characteristics of the nanometals have been proven to exert an effect on their antimicrobial activities. The positive surface charge of the metal nanoparticles facilitates their binding to the negatively charged surface of the bacteria which may result in an enhancement of the antimicrobial activity (Dutta et al. 2012; Dizaj et al. 2014; Tee et al. 2016; Raghunath and Perumal, 2017). The mechanism of antimicrobial action of metals and metal oxides is schematically represented in Fig. 5.6.

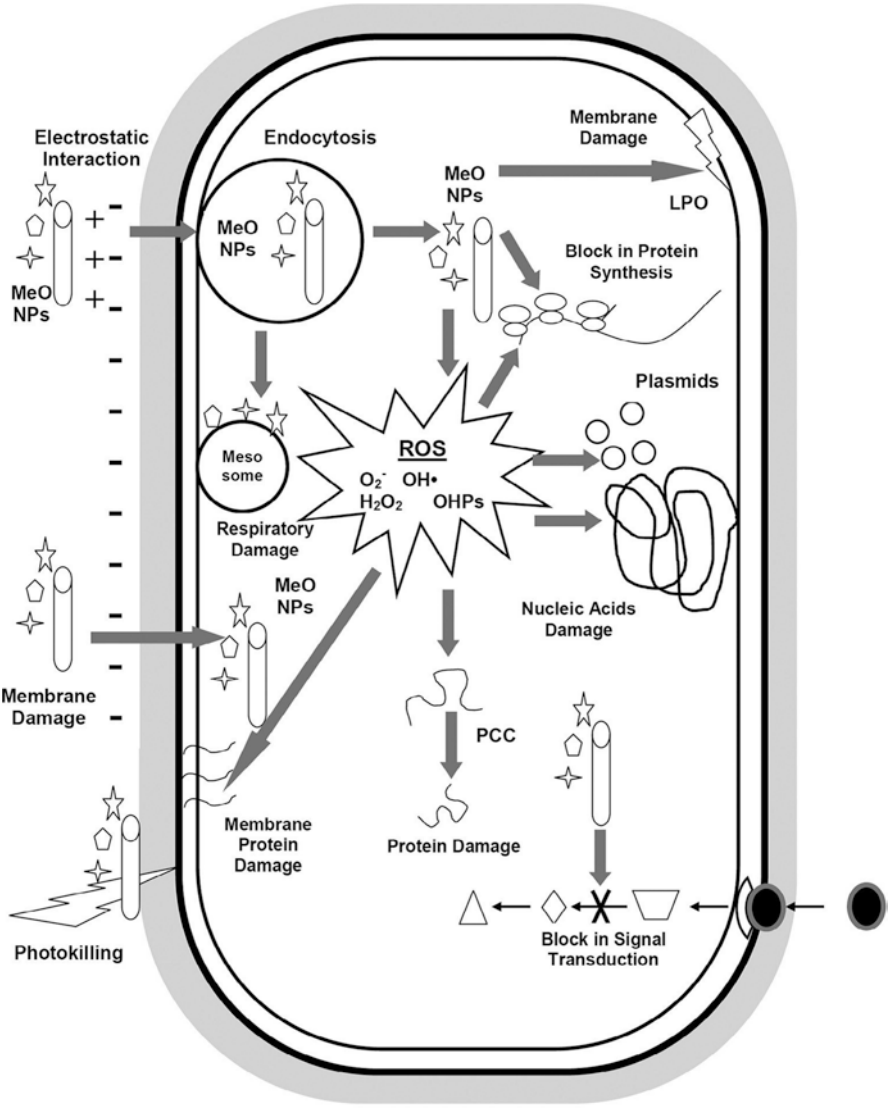

Fig. 5.6 An overview of the antimicrobial mechanism of inorganic nanoparticles. (Printed with permission from Raghunath and Perumal 2017)

Additionally, in the quest to fight AMR, inorganic nanomaterials have emerged as promising candidates since they possess greater durability, lower toxicity, higher stability and selectivity and above all their inhibitory effect against a wide range of multidrug-resistant strains (Pelgrift and Friedman, 2013). Moreover, the antifungal activity of gold, silver and zinc oxide nanoparticles was hugely effective in controlling the growth of *Aspergillus*, *Candida*, etc. (Nasrollahi et al. 2011; Wani and

Ahmad, 2013; Kairyte et al. 2013). In the fight against parasitic diseases such as malaria, leishmaniosis, schistosomiasis and toxoplasmosis, nanoparticles of silver, gold, titanium oxide, alumina, selenium and zinc oxide were able to control the proliferation and binding of the parasite to the host (Allahverdiyev et al. 2011a, 2011b; Soflaei et al. 2014; Marimuthu et al. 2011; Nadhman et al. 2014, Gogoi, 2017).

5.3.5 Carbon Nanostructures

Carbon nanostructures consist of many forms of nanocarbon that can be divided into three groups depending on their dimensions: (i) zero-dimensional (0D) such as fullerene, carbon dots, and nanodiamonds; (ii) one-dimensional (1D) such as carbon nanotubes (CNT), including single and multiwalled CNTs; and (iii) two-dimensional (2D) such as graphene and layered graphene sheets or nanoribbons (Aguilar, 2012). These carbon nanostructures find application in different emerging areas due to their unique properties and are known to exhibit significant antimicrobial properties (Dizaj et al. 2014).

Fullerenes are spherical cage-like nanostructures made exclusively of carbon atoms (e.g. C60, C70). Their unique hollow shape and structural analogy with cellular vesicles make it an excellent drug delivery agent (Tripathi et al. 2015). Fullerenes display diverse biological activity, which arises from the fact that it can act either as an electron acceptor or donor. Fullerenes when irradiated with ultraviolet or visible light can convert molecular oxygen present within the cells into highly reactive singlet oxygen that can damage cellular membranes, inhibit the activity of various enzymes or may even lead to DNA cleavage. The photodynamic therapy (PDT) induced by fullerenes conjugated with photosensitizers had been exploited to control the growth of a broad spectrum of bacteria and fungi (Huang et al. 2010). For example, the cationic-substituted fullerene derivative when illuminated with white light effectively killed gram-positive (*S. aureus*), gram-negative bacteria (*E. coli*) and fungus (*C. albicans*) (Mizuno et al. 2011). A similar effect was reported with fullerenes bearing cationic charges from the addition of potassium iodide and irradiated with ultraviolet A (UVA) or white light killing *A. baumannii*, methicillin-resistant *S. aureus* and fungal yeast *C. albicans* in infected mouse (Zhang et al. 2015). The fullerene-mediated PDT of mice infected with *P. mirabilis* revealed 82% survival compared to 8% survival without treatment, whereas mice infected with highly virulent *P. aeruginosa* survived up to 60% when PDT was combined with an antibiotic, tobramycin (Lu et al. 2010). It has also been found that fullerene PDT is effective in healing wounds infected with pathogenic gram-negative bacteria (Sharma et al. 2011). Functionalized fullerenes with polycationic conjugates and stable synthetic bacteriochlorins allowed PDT to treat infections in animal models (Hamblin 2016). Additionally, biocompatible composites containing polysaccharides (cellulose, chitosan and γ-cyclodextrin) and fullerene derivatives substantially increased the composite's ability to reduce the growth of antibiotic-resistant bacteria such as vancomycin-resistant *Enterococcus* (Duri et al. 2017) (Fig. 5.7).

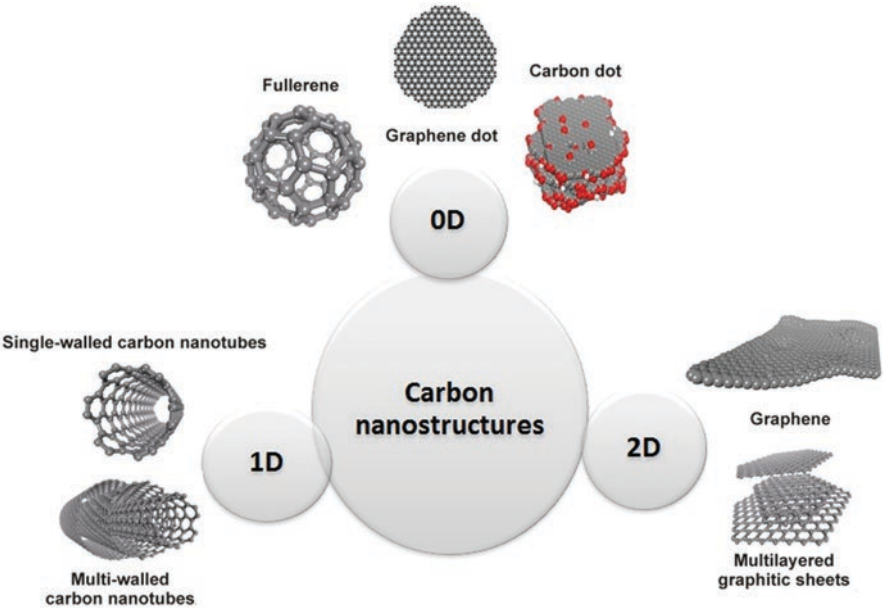

Fig. 5.7 Carbon nanostructures based on their dimensions

Carbon nanotubes (CNTs) are nanosized hollow cylindrical form of carbon formed by a single cylindrically shaped graphene sheet (single-walled carbon nanotubes, referred usually to as SWCNT) or several graphene sheets arranged concentrically (multiwalled carbon nanotubes, referred to as MWCNT). The antimicrobial activity of SWCNTs is attributed to severe membrane damage that leads to cell death. Studies revealed that SWCNTs proved to be potent bactericidal against gram-positive and gram-negative bacteria than MWCNTs because SWCNTs could penetrate into the cell wall better than MWCNTs due to their smaller diameter which initiated better interaction with the cell surface (Kang et al. 2007; Yang et al. 2010; Dong et al. 2012). CNTs coated with silver exhibited antimicrobial activity against mucoid and nonmucoid strains of *P. aeruginosa*. The mechanism of bactericidal effect was attributed to cell membrane integrity, downregulation of virulence-gene expression and induction of oxidative stress (Dosunmu et al. 2015). Additionally, improved bactericidal activity of PEGylated silver-coated SWCNT than their non-PEGylated counterparts was reported against *Salmonella enterica* serovar *Typhimurium* (Park et al. 2018). However, MWCNTs coated with silver and iron nanoparticles proved to be effective antimicrobial in water treatment (Ali et al. 2017) and the composites of lignin MWCNTs with polyvinyl alcohol for applications in wound dressings, scaffolds and antimicrobial textiles (Lee et al. 2018).

Compared with fullerenes and CNTs, graphene (an atom-thick sheet of graphite) and graphene oxide (an oxidized form of graphene) nanosheets present extraordi-

nary physicochemical properties which are responsible for their antimicrobial activities. The inhibitory effect of graphene on bacteria *E. coli, S. aureus* and *P. aeruginosa* and fungi *C. albicans*, *Aspergillus niger* and *A. flavus* has been reported in several studies (Palmeiri et al. 2018; Nguyen et al. 2019). Moreover, the photothermal effect of graphene oxide (GO) as antibacterial (against *S. aureus, P. aeruginosa*), antifungal (against *Saccharomyces cerevisiae* and *Candida utilis*) and in controlling the wound infection using near-infrared laser was also investigated and demonstrated promising result (Khan et al. 2015). Though the antimicrobial efficacy of graphene and GO is impressive, it is found to be toxic to mammalian cells. In order to reduce toxicity and increase the efficiency of GO, surface modification and functionalization with inorganic nanostructures, biomolecules and polymers are done and found to be effective against multidrug-resistant bacteria (Yousefi et al. 2017). The synergistic effect of nanomaterials such as metals, metal oxides and polymers with graphene-based nanostructures for stability and biocompatibility has a wide range of applications in antibacterial packaging, wound dressing and water disinfection (Ji et al. 2016). GO nanocomposites with metallic nanoparticles such as Ag, Au, Cu, Mg and Fe exhibit improved antibacterial as well as antifungal activity as compared to GO due to lower cytotoxicity (Li et al. 2013; Cui et al. 2014; Ji et al. 2016). Graphene nanocomposites containing poly-N-vinyl carbazole (PVK) showed higher bacterial toxicity against gram-negative bacteria *E. coli* and *Cupriavidus metallidurans* and gram-positive bacteria *B. subtilis* and *Rhodococcus opacus.* The nanocomposite encapsulated the bacterial cells, which led to reduced microbial metabolic activity and cell death (Carpio et al. 2012). Graphene-based nanomaterials functionalized with metal nanoparticles, photocatalysts, polymers and biocidal compounds were tailored for antimicrobial activities, used for water disinfection and for the development of antimicrobial polymeric membranes (Zhu et al. 2017). A potent bacterial effect was also reported when metal oxide nanoparticles were grown on the surface of chitosan-modified GO (Chowdhuri et al. 2015) and with chitosan-iron oxide-coated GO nanocomposite hydrogel (Konwar et al. 2016). Recently, the antiviral effect of silver nanoparticle-modified GO nanocomposites against porcine epidemic diarrhoea virus (PEDV) prevented the entry of the virus into the host cells and enhanced the production of interferon-α (IFN-α) and IFN-stimulating genes (ISGs), which directly inhibit the proliferation of the virus (Du et al. 2018).

Though a thorough understanding of the antimicrobial mechanism of graphene-based nanomaterials is still in its infancy, the physicochemical interaction between graphene and microbes is proposed to fall under any of the three categories, namely, (a) nano-knives derived from the action of sharp edges, (b) oxidative stress-mediated with/without the production of reactive oxygen species and (c) wrapping or trapping bacterial membranes derived from the flexible thin-film structure of graphenes (Zou et al. 2016).

5.3.6 *Quantum Dots*

Quantum dots (QDs) are semiconductor nanostructure with diameters in the range of 2–10 nm. These nanostructures emit light of varied colours depending on their size and shape. Due to their glowing properties, QDs are commonly used in imaging, sensors and biology (Frecker et al. 2016). A good number of researches have also established QDs as antimicrobial agents. These include QDs of inorganic heavy metal origin such as cadmium tellurium (CdTe), cadmium selenide (CdSe), cadmium sulphide (CdS), zinc oxide (ZnO) and carbon dots (C-dots) and their functionalized derivatives. Antibacterial activity of CdTe, CdSe and CdS QDs against *E. coli* was reported in a number of studies (Lu et al. 2008; Li et al. 2009). These QDs were investigated to understand its antimicrobial property; experiments indicated that the QDs bind with bacteria and impair the functions of cell's oxidative system via reactive oxygen species (ROS)-mediated pathway and Cd^{2+} ion release. The ROS and released Cd^{2+} ions lead to downregulations of antioxidative genes, and decreases of antioxidative enzyme activities, oxidative damage of protein and lipid and glutathione depletion were responsible for the QDs' cytotoxicity (Lu et al. 2008; Li et al. 2009). Besides, CdTe, CdSe and CdS and ZnO QDs proved to be effective against *Listeria monocytogenes*, *Salmonella enteritidis* and *E. coli* when bound in polystyrene film or suspended in polyvinylpyrrolidone gel (Jin et al. 2009). Quantum-sized silver nanoparticles stabilized with polyvinylpyrrolidone (PVP) inhibited the growth of *C. albicans* that was resistant to conventional antifungal drugs (Selvaraj et al. 2014). Similarly, the germicidal effect of different QDs coated with indolicidin was observed against *S. aureus*, *P. aeruginosa*, *E. coli*, and *Klebsiella pneumonia* (Galdiero et al. 2016). Furthermore, the nanocomposites of QDs such as chitin-CdTe films and CdSe QD-ZnO exhibited excellent antibacterial activity against gram-positive and gram-negative bacteria (Wansapura et al. 2017; Mahmoodi et al. 2018). Research showed that conjugation of QDs with different nanomaterials enhanced their antimicrobial activity; for example, the germicidal action of MWCNTs was reported to be poor against different bacterial strains, but when MWCNTs were conjugated with CdS and Ag_2S QDs, its antimicrobial activity improved severalfold (Neelgund et al. 2012). Similarly, gold-carbon dot (Au-C-dot) nanoconjugate exhibited a profound effect on the susceptibility of a fungus, *C. albicans* (Priyadarshini et al. 2018).

C-dots, graphene and graphene oxide QDs (GOQDs) are known to be "safe" carbon nanomaterials and an effective antimicrobial agent. Their mechanism of microbial cell death is linked to the peroxidase-like activity that catalyzes the decomposition of H_2O_2, generating free radical, •OH. Since the •OH has higher antibacterial activity, the conversion of H_2O_2 into •OH improves the antibacterial performance. This property of graphene QDs is effective against both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria and in wound healing (Sun et al. 2014). The photoexcitation of graphene QDs (GQDs) leads to the generation of ROS which is found to inhibit *E. coli* and methicillin-resistant *S. aureus* (Ristic et al. 2014). GQDs doped with nitrogen and functionalized with an amino group

serving as a photosensitizer in photodynamic therapy had superior ability to generate ROS as compared to unmodified GQDs, which were able to completely eliminate multidrug-resistant species (Kuo et al. 2018). Similarly, sulphur- and nitrogen-doped C-dots demonstrated improved antibacterial activity against gram-negative, gram-positive and drug-resistant bacterial strains (Travlou et al. 2018). Moreover, antibiotic attached to C-dots proved to be an effective nanocarrier for controlled drug release and high antimicrobial activity against both gram-positive and gram-negative bacteria (Thakur et al. 2014). Similar antibacterial activity was observed against *P. aeruginosa* when C-dots were doped with gallium (Kumar et al. 2017). Antiviral activity of C-dots was achieved with surface functionalization with 2,2′-(ethylenedioxy)bis(ethylamine) (EDA) and 3-ethoxypropylamine (EPA). Both EDA and EPA C-dots effectively inhibited the binding of two strains of human norovirus-like particles (VLPs) to histo-blood group antigen (HBGA) receptors on human cells (Dong et al. 2017).

5.3.7 Electrospun Nanofibres

Polymer fibre materials that are shrunk from micrometre to submicron or nanometre scale show amazing characteristics such as large surface area to volume ratio, flexibility in surface functionalities and higher mechanical performance (stiffness and tensile strength). These superior properties make the polymer nanofibres (NF) optimal candidates for many applications such as filtration membranes, catalytic nanofibres, fibre-based sensors and tissue engineering scaffolds (Jayakumar et al. 2010; Ma and Hsiao 2018; Haider et al. 2018). In order to synthesize these nanofibres, several processing techniques such as drawing, template synthesis, phase separation, self-assembly and electrospinning have been used (Huang et al. 2003). Among these techniques, electrospinning has gained popularity recently due to the production of polymer fibres with diameters varying from 3 nm to 5 μm. Electrospinning provides multiple desirable features for wound dressings, including high absorptivity due to high surface-area-to-volume ratio, high gas permeation and conformability to a contour of the wound bed (Lalani and Lui 2012). The attractive feature of electrospinning is the simplicity and inexpensive nature of the setup; the typical electrospinning setup consists of a syringe pump, a high-voltage source and a collector. The working principle of electrospinning was nicely reviewed by Pham et al. (2006). This approach has been used successfully to spin a number of synthetic and natural polymers such as cellulose, poly(acrylonitrile), poly(caprolactone), poly(methyl methacrylate), poly(vinyl alcohol) and polyimide fibres into nanofibres applied in the fields of biomedicine (wound healing) and biotechnology (Haider et al. 2018).

The electrospun polymeric nanofibres loaded with silver (Ag) nanoparticles, chitosan and their composites have demonstrated excellent antimicrobial activity against bacteria, fungi and parasitic diseases. Electrospun antimicrobial polyurethane nanofibres containing Ag indicated high bactericidal effect against *E. coli* and

S. typhimurium (Sheikh et al. 2009). Nanofibre mats loaded with Ag nanoparticles (~25-nm diameter) enveloped in chitosan and cross-linked with glutaraldehyde showed superior properties and synergistic antibacterial effects (Abdelgawad, et al. 2014). The electrospun cellulose acetate containing Ag nanoparticles on their surface when irradiated with UV exhibited strong antimicrobial activity (Son et al. 2006). Similarly, the electrospun cellulose nanofibre mats decorated with silver ion inactivated *E. coli* (Reiger et al. 2016). Antimicrobial nanofibrous membranes developed from electrospun polyacrylonitrile nanofibres with diameters of ~450 nm loaded with Ag nanoparticles demonstrated a convenient and cost-effective approach to develop antimicrobial nanofibrous membranes that would be particularly suitable for the filtration of water and/or air (Zhang et al. 2011). Additionally, the chitosan-based nanofibres such as a mixture of poly(lactide-co-glycolide) (PLGA) and chitosan when electrospun yielded cylindrical and narrow-diameter (356 nm) polymeric fibres. The PLGA-chitosan mats were then functionalized with graphene oxide and decorated with silver nanoparticles, effectively inactivating both gram-negative (*E. coli* and *P. aeruginosa*) and gram-positive (*S. aureus*) bacteria (De Faria et al. 2015). Chitosan nanofibres electrospunned with poly(ethylene oxide) and silver nitrate, as a co-electrospinning polymer and silver nanoparticle precursor, revealed antibacterial activity (Annur et al. 2015), Similarly, the electrospun fibrous membrane of zwitterionic poly(sulfobetaine methacrylate) (PSBMA) known for its superhydrophilic and ultralow biofouling properties makes it a promising material for superabsorbent and non-adherent wound dressings. Bacterial adhesion studies using gram-negative *P. aeruginosa* and gram-positive *S. epidermidis* showed that the PSBMA electrospun membrane was highly resistant to bacterial adhesion. Moreover, the Ag-impregnated electrospun PSBMA membrane proved microbicidal against both *S. epidermidis* and *P. aeruginosa* (Lalani and Lui 2012). Furthermore, the antimicrobial peptide pleurocidin is known for broad microbial inhibition and thermal/pH tolerance when incorporated with poly(vinyl alcohol) electrospun nanofibre showing higher inhibition efficiency than free pleurocidin against *E. coli* (Wang et al. 2015).

However, for fungal infections, clotrimazole-loaded microemulsion (a mixture of polyvinyl alcohol and chitosan) containing nanofibre mats demonstrated mucoadhesive properties against oral candidiasis and is now developed as an alternative for oral applications (Tonglairoum et al. 2015). Polylactic acid films coated by electrospinning with a formulation containing chitosan demonstrated excellent antifungal activities against *Aspergillus brasiliensis*, *Fusarium graminearum*, *Penicillium corylophilum* (Mitelut et al. 2017). Similarly, electrospun poly(lactic acid) (PLLA) nanofibre membranes loaded with bovine lactoferrin (bLF) membranes display antifungal activity against *A. nidulans* by inhibiting spore germination and mycelial growth (Machado et al. 2018). Moreover, the sustained release of a cellulose acetate solution containing artemisinin, an antimalarial drug, developed from electrospinning of poly(vinyl pyrrolidone) confirmed the higher bioactivity of the released drug from the composite (Shi et al. 2013). Recently, electrospun core/shell nanofibres containing different percentages of artemisinin were developed as new systems for drug administration in malaria. The core consisted of hyperbranched

poly(butylene adipate) and poly(vinylpirrolidone) as shell material, and a controlled proliferation of malarial parasites (*P. falciparum*) was reported in this study (Bonadies et al. 2017)

Though electrospinning is well known for its simplicity and cost-effective setup, its disadvantage lies in the production of fine fibres and low yield (Sarkar et al. 2010). A recently developed method known as Forcespinning® (FS) has shown the capability to produce fine fibres from melt and solution through centrifugal spinning (Padron et al. 2013). The FS method does not require electricity and broadens the choice of materials to be spun into fibres (Padron et al. 2013; Rane et al. 2013). The process is highly controllable at the industrial scale and has shown production rates of up to hundreds of metres per minute. Previous FS studies have successfully produced wound dressings composed of cellulose acetate fibres embedded with silver nanoparticles (AgNPs) and ternary composite fibre dressings such as pullulan/tannic acid/chitosan fibre and polyvinyl alcohol/chitosan/tannic, all of which showed antimicrobial activity (Xu et al. 2015, 2016). Recently, chitosan binary nonwoven fine fibre composite scaffolds composed of chitosan/cinnamaldehyde (CA) and chitosan/AgNPs were produced using FS technology. Cinnamaldehyde and silver are known to possess strong antimicrobial properties and therefore its effect in these binary composites exhibited improved antimicrobial activity against *S. aureus* (Cremar et al. 2018).

5.3.8 Other Potential Nanomaterials Effective Against Microorganisms

Continuous research for the development of new nanomaterials that are potent antimicrobial agent has grown severalfold. Many nanomaterials such as nanodiamonds, nanoribbons, nanopowders and nanoclays have shown their advantages over existing nanomaterials against infectious diseases as well as against multidrug-resistant (MDR) species. For example, the advantage of nanodiamonds (diamond nanoparticles) is that they are completely inert, optically transparent and biocompatible as compared to other carbon-based materials such as fullerenes and carbon nanotubes. Although the *in vivo* toxicity of nanodiamonds (ND) against bacteria and biofilm formation depends on their surface characteristics (Wehling et al. 2014) and functionalization (Turcheniuk et al. 2015), they have been found to be non-cytotoxic to a variety of cell types and have been thus used in a number of biomedical applications (Liu et al. 2007; Marcon et al. 2010; Mochalin et al. 2012). Moreover, functionalized NDs with hydroxyl, amine, carboxyl, saccharides, etc. have found to be an effective antimicrobial and antibiofilm agent (Khannal et al. 2015; Szunerits et al. 2016). The powdered nanoparticles (nanopowder) of Au, Ag, Al_2O_3, Co_3O_4, CuO, Fe_2O_3, Fe_3O_4, MgO, ZnO, NiO SiO_2, graphene, etc. and their doping on hydroxyapatite powders have demonstrated pathogenic effect against bacteria and fungi (Sygnatowicz et al. 2010; Stanić et al. 2010; Marriappan et al. 2017). The

nanopowders were obtained by conventional techniques such as nanoprecipitation, emulsion-diffusion and double emulsification, but recently, with the emergence of electrospraying technique, developing micro- and nanosized particles containing bioactive compounds is booming. Electrospraying improved nanoparticle production such as scalability, reproducibility and encapsulation with biodegradable polymers obtained from food products (proteins, carbohydrates), such as chitosan, alginate, gelatin, agar, starch or gluten (Tapia-Hernandez et al. 2015). Thus, electrosprayed nanoparticles and nanofibres are both employed as natural or synthetic carriers for the delivery of entrapped drugs, growth factors, health supplements and vitamins and as antimicrobial agents (Sridhar et al. 2015; Rodríguez-Tobías, et al. 2016).

Furthermore, nanoclays (nanoparticles of layered mineral silicates) have also been found to be of good importance in polymer nanocomposites and as drug delivery carriers. Depending on the chemical composition and nanoparticle morphology, nanoclays such as commercially available montmorillonite and naturally occurring cloisite have been effective against gram-positive and gram-negative bacteria (Hong and Rhim, 2008). A similar and improved efficiency of cetyltrimethylammonium bromide (CTAB)-modified montmorillonite with poly(butylene adipate-co-terephthalate) nanocomposite films and organo-modified Algerian montmorillonites with poly(ε-caprolactone) was reported to be biodegradable (Mondal et al. 2014; Yahiaoui et al. 2015). The incorporation of biodegradable natural and polymeric materials with nanoclays and their ability to retard microbial spoilage makes them an ideal material for food packaging (Mondal et al. 2014; Jiménez et al. 2016).

5.4 Potential Toxicity of Nanomaterials

Advancement in nanoscience and nanotechnology led to the development of nanomaterials and nanostructures which have been seen as novel alternatives to antibiotics in infectious diseases. However, these nanomaterial-based antimicrobial agents suffer from potential biological toxicity, poor degradation and other secondary pollution. For example, most of the semiconductor QDs made of heavy metal ions (e.g. Cd^{2+}) are responsible for their potential toxicity and their practical applications. Studies on a series of aqueous synthesized QDs, i.e. CdTe, CdTe/CdS core-shell structures and CdTe/CdS/ZnS core-shell-shell structures, revealed cytotoxicity is caused by an increase in the intracellular level of Cd^{2+} ions released from the QDs (Chen et al. 2012). The knowledge on the potential application of some of the metal oxide nanoparticles such as CuO, ZnO, Sb_2O_3, Mn_3O_4 and Co_3O_4 is limited because of their toxicity to mammalian cells at higher concentrations (Gajewicz et al. 2015; Ivask et al. 2015; Hou et al. 2018). It has been proposed that functionalization, ion doping and polymer conjugates of these metal oxide nanoparticles could be helpful to decrease the associated toxicity. Additionally, the toxicity of CNT samples was found to be dependent on its composition along with its geometry and surface functionalization. Several studies have suggested that well-functionalized CNTs are safe

to animal cells, while raw CNTs or CNTs without functionalization show severe toxicity to animal or human cells at even moderate dosage (Khalid et al. 2016). Other nanomaterials such as dendrimers, C-dots and fullerenes have been found to be cytotoxic. According to the reports, neurological and respiratory damage, circulatory problems and some other toxicity effect of nanoparticles are the main concerns with the use of nanoparticles (Elsaesser and Howard 2012; Dijaz et al. 2014). However, several types of nanoparticles such as TiO_2 and ZnO appear to be non-toxic with beneficial health effects; hence, few have been approved by the Food and Drug Administration and are commercially available (Elsaesser and Howard 2012). The cytotoxicity of some nanomaterials demands further research in functionalization and require alternative synthesis processes such that they are harmful to the microbes and not to the mammalian cells. The most common methods for nanoparticle synthesis were chemical and physical that is costly and potentially harmful to the environment. An alternative approach known as "green synthesis" is actively pursued nowadays for an efficient, inexpensive and environmentally safe method for producing nanoparticles with specified properties that are biocompatible and degradable (Marakov et al. 2014; Praveen et al. 2016). The area of green synthesis is rapidly gaining importance due to its growing success and ease of formation of nanoparticles. Presently, the potential of bio-organisms ranges from simple prokaryotic bacterial cells to eukaryotic fungus and even plants.

5.5 Conclusion and Future Prospects

Nanomaterials are showing promising solutions against infectious diseases due to their peculiar size, shape, chemical composition, surface structure, charge, solubility and their interactions with biomolecules and cells. It is well known that biological transport processes, anatomically and down to the cellular and subcellular levels, are affected by the physical attributes of the nanoparticles, including their size, shape and flexibility, as well as their chemical characteristics, including the presence of active ligands for recognition by and triggering of biological receptors. Therefore, it is of critical importance to utilize procedures that prepare nanostructures with high degrees of uniformity and with control over their physical and chemical traits. Though nanomaterials have excellent therapeutic importance, they suffer from the disadvantages of high cytotoxicity, biodegradation or agglomeration which is a major concern. Thus, understanding the nanoparticle and biological interface/interactions though complicated is very essential, especially considering the toxicity fears that currently exist in the field of nanomedicine field. There is a need for a set of design controls to study the nano-biointeractions including studies comprising of both the material properties and biological compositions such as analysis of transport kinetics, clearance, gene expression variations, chemical functionality, surface charge, biomolecular signalling and toxicity. Mostly, inorganic nanoparticles and dendrimers suffer from this problem. Thus, there exist opportunities in tailoring these nanoparticles such that minimum harm is caused to the human cells

without losing their antimicrobial effect. Another area of concern is the stability of the nanomaterials in biological fluids and to withstand the acidic pH of the stomach when administered orally. Liposomes and dendrimers are also susceptible to enzymatic degradation in the gastrointestinal tract. Here the needs of nanocapsules which can withstand the acidic pH are in demand for oral administration. These formulations should be mechanically and sterically stable such that they can survive these conditions and deliver the encapsulated drug via the normal absorption process. Additionally, the passage of therapeutic agents across the blood-brain barrier in neurological infections is a great challenge which can be accomplished by the use of nanomaterials. Nanomaterials can be engineered for treating diseases such as cerebral malaria, meningitis and encephalitis. Recent research in the field of multimetal oxides still demands extensive exploration since the combined effect of two or more particles can be better. Moreover, different nanomaterials are yet to be explored against infections of bacteria, fungi, viruses and parasites, where some may be more effective and safe than the one existing at present. To conclude, it can be stated that the application of nanomaterials against diseases is enormous with innumerable options of synthesizing and tailoring the particles. In view of designing these particles against different diseases, the most important concern must be that it should be safe for its therapeutic application in humans with minimum side effects.

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Chapter 6
Advanced Nanostructures for Oral Insulin Delivery

Chinnu Sabu and K. Pramod

Contents

Abstract 1. Issues: Oral insulin therapy is an efficient approach for the treatment and management of Type I and Type II diabetes. Extensive research has been carried out for oral delivery of insulin. The various physicochemical concerns affecting the permeability and dissolution are physical and chemical barriers, solubility, molecular weight, and partition coefficient. Oral insulin mimics the endogenous pathway of insulin; it suffers from first pass metabolism. The advances in nanomedicine result in a more robust insulin delivery system. The utilization of nanoparticles pos-

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sesses advantages like access to small areas of cell and small volume determination of the analyte.

2. Major Advances: The development of nanotechnology has resulted in a new approach to oral insulin delivery. Certain barriers exist in the mechanism of absorption of insulin which can be overcome by nanostructured insulin delivery. We reviewed the recent advances in nanostructured insulin delivery systems. Liquid crystalline nanoparticles, molecularly imprinted hydrogels, lipid-based carriers, polymeric carriers, iron oxide nanoparticles, gold nanoparticles, and silica nanoparticles are among the advanced oral insulin delivery systems. Nanostructures using the natural polymers like chitosan, dextran, and alginate are also discussed. All the developed system proves to a promising approach in the oral delivery of insulin. Biomimetic molecularly imprinted polymer (MIP) nanoparticles act as a potential form of oral insulin delivery system due to specificity and selectivity of the imprint to the polymer, whereas liquid crystalline nanoparticles act as thermodynamically stable structure in oral insulin delivery. Various nanostructures under development are covered in this chapter.

Keywords Oral insulin · Diabetes · Nanotechnology · Nanoparticles · Biomimetic · Polymers · Chitosan · Alginate · Liquid crystalline · Silica

Abbreviations

AuNP	gold nanoparticles
DPPC	dipalmitoylphosphatidylcholine
DTPA	diethylenetriaminepentaacetic acid
g-PGA	poly-g-glutamic acid
LSC	lauroyl sulfated chitosan
MBA	N,N-methylenebisacrylamide
MIP	Molecularly imprinted polymer
N-glut-PE	N-glutaryl-phosphatidylethanolamine
PLGA	poly(lactide-co-glycolide)
PLGA-PEG	poly (D, L-lactic-co-glycolic acid)-polyethylene glycol
SLN	solid lipid nanoparticles
TMC	N-trimethyl chitosan chloride
TMC-Cys	trimethyl chitosan-cysteine

6.1 Introduction

Diabetes is a metabolic disorder characterized by elevated blood glucose level and an inability to regulate the blood glucose level homeostasis (American Diabetes Association 2010). Diabetes has become a global disorder affecting millions of people worldwide and is accustomed to be the major reason of death by 2030. Type 1 diabetes is distinguished by the inability to produce insulin due to autoimmune destruction of beta cells, whereas Type 2 diabetes results from insulin resistance or deficiency of cellular response to insulin in the bloodstream (Ross et al. 2004). Therefore, insulin is essential to control the blood glucose level. The prevailing standard treatment for Type 1 and Type 2 consists of subcutaneous insulin injections (Mo et al. 2014).

The different routes of insulin absorption like intradermal, intramuscular, and intravenous were found to be nonreliable for frequent administration of insulin. Oral insulin delivery points to deliver insulin with more patient compliance. The advances in nanotechnology have enhanced the fabrication of novel glucose measurement and insulin delivery systems, improving the quality of life for diabetics (Disanto et al. 2016). The long-term exposure to excess insulin results in alteration of cell division process (Ahmad et al. 2012). Therefore, a tight control of insulin is required to prevent insulin overdose. The incorporation of nanoparticles shows increased sensor sensitivity and temporal response. Moreover, the utilization of nanoparticles possesses advantages like access to small areas of cell and small volume determination of the analyte. Indeed, advances in nanomedicine result in a more robust insulin delivery system (Mo et al. 2014). The traditional delivery requires frequent administration of insulin, resulting in decreased patient compliance, pain, tissue necrosis, infection, and nerve damage.

6.2 Oral Insulin Delivery

The oral route is the mostly preferred route of drug administration. Insulin is delivered directly to the liver, generating high portal systemic gradients. Thereby, it mimics the endogenous secretion of insulin. Poor bioavailability is the main problem considering oral insulin delivery. The various physicochemical concerns affecting the permeability and dissolution of gastrointestinal barrier are physical and chemical barriers, solubility, molecular weight, and partition coefficient. Nanoparticles allow the formulator to design various release profile and help to achieve local or systemic targeting of the encapsulated drug (Matteucci et al. 2018). However, transfer of significantly high quantity of insulin through the intestine leads to certain adverse effect. This problem can be overcome by using carriers for better performance (Nur and Vasiljevic 2017).

6.2.1 Merits and Demerits of Oral Insulin Delivery

Although various works have been carried out in insulin delivery, diabetes fails in long-lasting treatment due to side effects. Therefore, improvement is a need for successful delivery of insulin. The concern is regarding the route of administration. Parenteral administration of insulin is obstructed by the lack of patient compliance due to the painful administration of needed. However, the oral route of insulin is considered as a most effective route of administration due to its convenience of self-medication (Fonte et al. 2015). The dose of insulin can be varied in response to efficacy and toxicity in individual patients. Since oral insulin mimics the endogenous pathway of insulin, it suffers from first pass metabolism. Indeed, subcutaneous administration of insulin results in a low first pass effect (Plapied et al. 2011).

The oral route of administration helps in the avoidance of allergic reactions, lipodystrophy, and risk of disease transmission. Moreover, the oral route is considered as more effective since it does not need any support or specialized persons. Also, it reduces the number of visits to the hospital and cost of injections (Pridgen et al. 2014). However, the development of a productive oral insulin formulation is still challenging. Various barriers need to be overwhelmed for the efficient delivery of insulin across the gastrointestinal tract. One of the major factors governing the passage of the drug through the intestinal barrier is the size of the molecules. Large molecules find difficult to absorb, whereas smaller molecules will be taken by cell in conjugation with protein transporters. This size restriction is enabled by mechanical barrier of the intestine made up of mucous, cell membrane, and tight junctions. Another barrier that plays a major role is the hepatic barrier which comprises of various metabolic enzymes. Chemical barrier such as pH inactivation hinders insulin absorption. Further pH various causes bond cleavage leading to protein inactivity. The pH of the stomach, enzymatic activity, and poor permeability are some of the major factors concerning the oral insulin delivery (Fonte et al. 2015).

6.2.2 Role and Mechanism of Nanocarriers in Oral Insulin Delivery

The bioavailability of an orally delivered drug is mainly affected by the physicochemical characteristics of the drug. The absorption of the drug across the gastrointestinal tract occurs at different sites based on their size (Sharma et al. 2015). Particles with 1 μm are absorbed by the mechanism of phagocytosis by intestinal macrophages, whereas particles with size less than 10 μm are transported through Peyer's patch of the gastrointestinal tract. The mechanism of absorption of particles with 200 nm occurs through endocytosis by enterocytes (Hagan 1996). The presence of glycoprotein on enterocytes surface renders low systemic bioavailability of drug, affecting the absorption and excretion of drugs (Varma et al. 2003).

A promising nanomedicine must be stable, biodegradable, nontoxic, noninflammatory, non-thrombogenic, and non-immunogenic. Moreover, it should be easily removed by the reticuloendothelial system (Kumari et al. 2010). Nanostructured systems for oral delivery of insulin possesses certain advantages like enhanced efficacy, tolerability, and specificity (Hall et al. 2007). Nanostructured oral drug delivery systems help in the delivery of poorly water-soluble drugs, transcytosis of the drug across intestinal barrier, and intracellular delivery of macromolecules. One of the disadvantages of oral delivery is the poor absorption from the gastrointestinal tract. Nanoparticles improve the oral delivery of insulin by promoting insulin uptake by the transcellular or paracellular pathway.

6.3 Nanocarrier-Mediated Oral Insulin Delivery

The drawback associated with conventional injections could be overcome by the use of nanocarriers for delivery of insulin. Nanoparticles possess the ability to preserve insulin from digestive enzymes in the gastrointestinal tract and allow the movement of macromolecules across the desired site along the gastrointestinal tract (Paul et al. 2017). The oral route is considered more acceptable since it resembles endogenous insulin pathway. Among nanoparticles, the biopolymer-based nanoparticles have gained much interest due to biocompatibility and biodegradability.

Advances in nanotechnology results in increased cellular uptake of insulin. Natural and synthetic polymers were utilized to formulate nanoparticle-based drug delivery system. However, cytotoxicity and immunological response need to be assessed to ensure the safety of nanoparticles. Accumulation of nanoparticle inside the cell can cause toxicity at cellular levels. Formulation of liposome protects the drug from degradation in the gastrointestinal tract. Solid lipid nanoparticles possess high tolerability and increased bioavailability. The application of nanotechnology in oral insulin delivery results in several advantages. Firstly, the nanoparticle shields the entrapped drug from the harsh environment of the gastrointestinal tract. This helps to reach the desired site of administration. Nanostructured oral insulin delivery enhances the water solubility of the drug. They increase the intestinal solubility of the drug. The particles are easily taken up by microfold cells which show high transcytotic capacity and low lysosomal hydrolase activity. Moreover, the nanostructured oral insulin delivery reduces the dosing frequency, resulting in the controlled or sustained release of the nanoencapsulated drug (Diab et al. 2012).

6.3.1 Insulin-Imprinted Polymeric Nanoparticle

Biomimetic molecularly imprinted polymer (MIP) nanoparticles act as a promising form of oral insulin delivery system by creating a well-defined structure with nanosized cavities, resulting in cross-linked functional monomers. The system elicits

unique features like robust physical structures, ease of preparation, biocompatibility, and loading capabilities (Paul et al. 2017). MIP results in nanoparticle–biomolecule association leading to the interaction between the initial templates which produce multiple binding sites and further stimulate biological recognition. The usage of molecular imprinting on polymeric nanoparticles can be achieved with the help of biomimetic carriers (Zaidi 2016). However, one of the major advantages associated with MIP by precipitation polymerization is the creation of a selective nanoscale environment, resulting in enhanced affinity of the specific functional group at recognition sites.

Insulin administered by subcutaneous route results in poor patient compliance. Therefore, oral delivery of insulin results in the appropriate release of insulin in the gastrointestinal tract for an extended period of time. The molecules and receptors present in islets act as imprinting templates for insulin binding. MIP results in the development of recognition sites for binding to islet's cell membrane. N,N-methylenebisacrylamide (MBA) nanoparticles prepared from multifunctional monomer act on MIP binding sites. The cellular uptake into systemic circulation is affected by the property of template imprinting into polymer nanoparticle. Further, the selectivity and specificity of the imprint of the polymer depend on the ratio of functional monomer and cross-linker (Zaidi 2016).

Recently, studies have been conducted on MBA cross-linked nanoparticles comprising various functional groups that interact with the template. During the polymerization procedure, the adhesive characteristics of MBA functional monomer result in the attachment onto the cross-linked chains of insulin–MIPs. The polymer precipitation method offers the ease of preparation without the addition of surfactants or stabilizers. Polymerization results in the formation of individual particles through non-covalent interactions by specific functionality through the nanoparticle–protein association which further leads to improved protein loading and delivery efficiency (Paul et al. 2017).

6.3.2 pH-Sensitive Insulin-Loaded Nanohydrogel

Smart polymers are soluble, surface-coated, or cross-linked polymers showing large and sharp physical and chemical changes in response to stimuli such as temperature, pH, solvent composition, and electrical fields. Hydrogels are three-dimensional cross-linked macromolecular polymer networks possessing the ability to swell in an aqueous environment in response to environmental stimuli (Sahiner et al. 2006). Among the hydrogels, pH-sensitive nanohydrogel is a promising drug carrier due to its response to environmental stimuli (Chen et al. 2006). Studies have shown that insulin attached in nanohydrogel in 1,4-dioxane solvent has low release and absorption in the acidic condition of the gastrointestinal tract. This is because the formation of intermolecular complex takes place in the acidic condition of the stomach and insulin is trapped inside the polymer network complex by modified double emulsion method. Thereby, the trapped insulin was pro-

tected from proteolytic enzymes. The complex gets to break down in neutral and alkaline intestinal environment (Journal et al. 2016).

6.3.3 *Polymeric Nanoparticles and Micelles*

Polymeric nanoparticles and micelles are a category of nanostructured carriers for oral delivery of insulin. In case of nanospheres, the drug is uniformly dissolved or dispersed in polymer matrix, whereas a nanocapsule constitutes a vesicle in which drug core is enclosed by a polymeric film. Self-aggregation of amphiphilic polymers to nanosized aggregates leads to the development of micelles. The core of the micelles is constituted by hydrophobic moiety, and the corona in the shell of micelles forms the hydrophilic moiety. The mechanism of drug delivery by polymeric nanoparticles and micelles mainly takes place by endocytosis which further depends on the surface property of nanocarriers (Sadashiv et al. 2015).

Modification of the surface and enteric coating of the nanoparticles enhances the gastrointestinal absorption of insulin. Various other strategies to improve the oral absorption of insulin include the use of enzyme inhibitor and absorption enhancer. Synthetic or natural polymeric nanoparticle is a method to enhance gastrointestinal absorption of insulin by modulating insulin release and subsequently its therapeutic property. Insulin-loaded nanoparticles synthesized by biodegradable polymers are absorbed via intestinal epithelial cells and transport insulin via intestinal mucosa (Woitiski et al. 2008). The gastrointestinal absorption can be further enhanced by the co-administration of permeation enhancers that widen intracellular junctions (Iyer et al. 2010). Permeation enhancers like fatty acids, surfactants, Ca^{2+}-chelating agents, and *Zonula occludens* toxin are usually introduced into the formulation. Fatty acids enhance drug absorption by the transient opening of the tight junctions. Surfactants promote transcellular transport by disrupting the lipid bilayer. Chelating agents and *Zonula occludens* toxin alter the tight junction to increase the absorption of insulin (Park et al. 2011).

Enteric-coating approach is another way to increase the oral absorption of insulin by a pH-dependent mechanism. This approach is mainly applicable for polyacrylic polymers and cellulosic polymers (Su et al. 2012; Chen et al. 2012). The enteric coating protects insulin from the gastric acidic fluids and rapidly liberates insulin in the proximal segment of small intestine. Moreover, it increases the absorption and relative bioavailability. On the other hand, the enzyme inhibitor approach improves the systemic bioavailability of insulin by inhibiting the activity of gastric enzymes by protease inhibitor. The protease activity can also be inhibited by introduction of cationic metal chelating agents like diethylenetriaminepentaacetic acid (DTPA) (Su et al. 2012). The chelating agents provide a protective effect by binding to cofactors of the enzyme system leading to structural changes and lack of enzymatic activity.

The various parameters that improve the encapsulation efficiency of insulin include pH of the aqueous-phase insulin solution, the origin of insulin monomer, and insulin concentration. The surface characterization of nanoparticle can be

enhanced by using different polymer groups or by conjugating polymer to nanoparticle surface. The application of targeting ligand to nanoparticle surface can enhance the interaction with nanoparticle surface (Pridgen et al. 2015).

In the oral delivery of insulin, the copolymers used to form micelles should possess certain characteristics like fast self-aggregation in water, stability in the gastrointestinal tract, biocompatibility and non-toxicity, and easy synthesis on large scale. Stable micelle complex can be formed by incorporation of cross-linking hydrophobic groups into the hydrophobic polymer (Sadashiv et al. 2015). The micelle complex provides stability against aggregation. The permeation-enhancing property can be improved by conjugation of the functional moiety to nanocarriers. The hydrophilic portion of the copolymer is conjugated with the functional moiety. It is reported that an arginine-rich peptide-modified chitosan, N-octyl-N-arginine chitosan, improves the oral absorption of insulin (Zhang et al. 2013). The insulin absorption can also be enhanced by attachment of specific target ligand to the nanoparticle surface. This fact was proved by lectin-modified polystyrene nanoparticle-mediated insulin absorption. Recently, an oral insulin delivery system was fabricated based on a natural and dual functional delivery device based on chitosan and PLGA (Fig. 6.1) (Zhang et al. 2017).

The various physicochemical and biological properties affecting oral absorption of micelles and nanoparticles include particle size, surface charge, nanoparticle stability, the residence time of nanoparticles at the absorption site, and the intestinal contents (Bakhru et al. 2013). Generally, nanoparticles have greater cellular uptake efficiency than microparticles. The decrease in particle size to less than 1 μm increases the cellular uptake, while particles up to 100 μm uptaken by Peyer's patches are absorbed into systemic circulation. The absorption across intestinal epithelium is affected by the surface charge of the particle. Therefore, the epithelium being negatively charged, positively charged particles are more readily absorbed than negatively charged or uncharged particles (Bakhru et al. 2013). Another factor affecting oral absorption is colloidal stability. Aggregation or flocculation results in colloidal instability (Florence 2012). It is an important factor for nanocarriers comprising specific targeting ligands; thereby, effective internalization can be achieved.

The increased surface area of the small intestine due to the presence of villi plays a great role in the gastrointestinal absorption of drugs. The nanoparticles and micelles are usually transported across intestinal epithelium via paracellular and transcellular transport mechanism (Florence 2004; Chen et al. 2011). The paracellular route is mainly preferred for the transport of hydrophilic drugs. But this pathway is not possible for particles larger than 1 nm due to small interstitial spaces and the presence of tight junction between epithelial cells (Chen et al. 2011). However, this is not possible for polymeric nanoparticles. Their transport can be facilitated by the use of permeation enhancers which reversibly open the tight junctions. Moreover, the mechanism is still impossible for particle larger than 20 nm (Sonaje et al. 2009). In this case, the nanoparticles need to be destabilized and disintegrated in the intercellular space when interacting with the tight junction. Chitosan is an example of permeation enhancer which increases the paracellular transport by the reaction of the positively charged polymers with the negatively charged cell membrane.

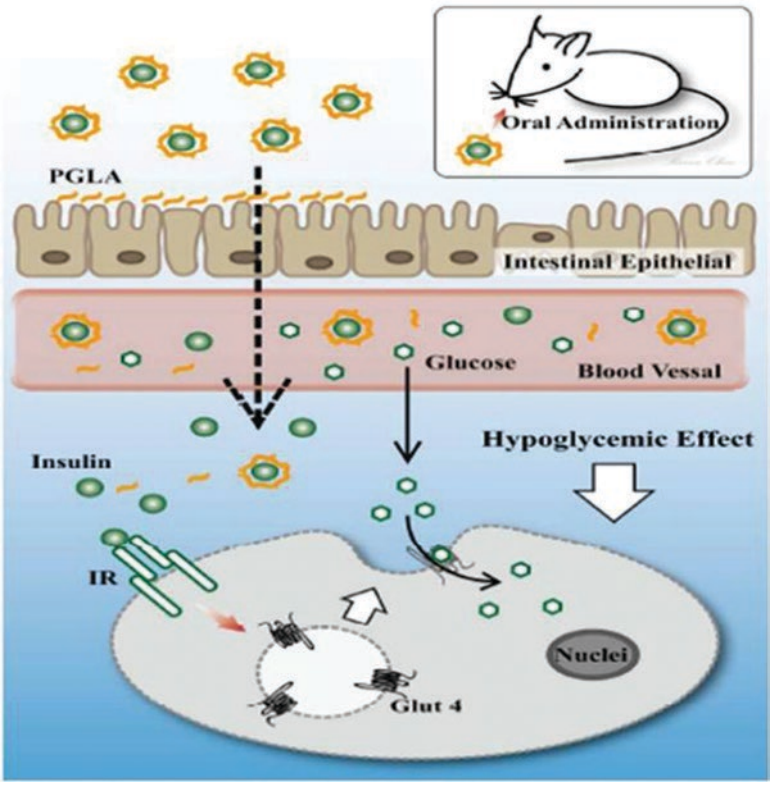

Fig. 6.1 Mechanism of absorption of insulin-loaded polymeric nanoparticle. When the insulin-loaded nanoparticle is transported across the intestinal epithelium and the blood vessels, the insulin is gradually released and interacts with receptor, resulting in hypoglycemic effect. ("Reprinted with permission from (Zhang et al. 2017). Copyright (2017) American Chemical Society")

Transcellular route of mechanism mainly takes place when the nanoparticles are taken by enterocytes or by the M cell of Peyer's patch avoiding the presystemic hepatic metabolism. Nanoparticles can be prominently transported via active transcellular pathway (Shahbazi and Santos 2013). The active transcellular process involves phagocytosis, macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis (Chen et al. 2011).

6.3.4 Amphiphilic Hollow Carbon Nanosphere

Recently, carbon nanomaterials have been used as novel biomolecular carriers since they possess the ability to transport covalently bonded drugs or molecular probes across the cell membrane. The poor penetration of the drug can be overcome by conjugation with a nanocarrier for maximum cellular uptake. Carbon

spheres are biocompatible and non-immunogenic agents that carry the active ingredient by the process of surface adsorption or deposition, pore filling, incorporation in the carbon matrix, and surface covalent coupling (Ganeshkumar et al. 2013). Hollow carbon nanospheres with the amphiphilic property of yeast can be used for oral delivery of insulin. This pH-sensitive coated hollow carbon nanospheres increase the intestinal absorption of insulin and reduce the level of blood glucose (Ganeshkumar et al. 2013).

6.3.5 Biodegradable Polymeric Systems

Natural Polymeric Systems

Chitosan is a natural polymer produced by deacetylation of chitin. It is a biocompatible, biodegradable, and protective polymer composed of glucosamine and N-acetylglucosamine (Mukhopadhyay et al. 2013). Recently, it is reported that insulin-loaded iron oxide–chitosan nanoparticles reduce the blood glucose level. This approach is useful in the drug delivery because of the magnetic property of iron oxide nanoparticles (Kebede et al. 2013). However, the insolubility of chitosan in neutral environment tends to lose charge which further results in loss of mucoadhesive property and tight junction opening activity (Qian et al. 2006). This problem can be overcome by the use of chitosan derivatives such as quaternized chitosan, thiolated chitosan, carboxylated chitosan, and amphiphilic chitosan. Currently, they are being evaluated for their potential for oral insulin delivery (Chen et al. 2013).

Unlike chitosan, quaternized chitosan regains its positive charge in the neutral environment leading to increased bioavailability and residence time (Yan and Ajun 2007). This is proved by N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride containing oral insulin delivery system which increased interaction with mucus (Sonia and Sharma 2012). However, the high positive charge of the quaternized ammonium compounds can cause toxicity to the cell membrane. Fortunately, N-trimethyl chitosan chloride (TMC) coated with polyethylene glycol shows reduced toxicity to the cell membrane (Prego et al. 2006; Zhu et al. 2007). Thiolated chitosan is more mucoadhesive than unmodified chitosan (Chen et al. 2013). Thiolated TMC was prepared to combine the mucoadhesion of TMC and the permeation-enhancing abilities of thiolated polymers for oral insulin delivery. Similarly, trimethyl chitosan-cysteine (TMC-Cys) shows enhanced absorption (Yin et al. 2009). The thiolated chitosan shows increased mucoadhesive property in the order chitosan thiobutylamidine with the highest followed by chitosan–4-mercaptobenzoic acid, chitosan–glutathione, chitosan–6-mercaptonicotinic acid, chitosan–N-acetyl cysteine, chitosan–thioglycolic acid, and unmodified chitosan (Mueller et al. 2011).

The water solubility of chitosan can be increased by modifying it with negatively charged groups, resulting in the formation of carboxylated chitosan (Liu et al. 2013). Carboxylated chitosan-grafted poly(methyl methacrylate) nanoparticles enhance the oral bioavailability of insulin (Cui et al. 2009). Nontoxic and increased

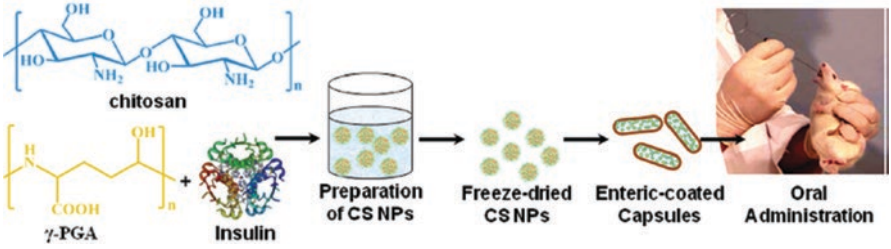

Fig. 6.2 Schematic representation of enteric-coated capsule with chitosan insulin nanoparticle for oral insulin delivery. The enteric-coated capsule prevents the release of insulin in the stomach. The oral bioavailability of insulin is enhanced by increasing the absorption of insulin in the small intestine. ("Reprinted with permission from (Sung et al. 2012). Copyright (2012) American Chemical Society")

mucoadhesive property for oral insulin delivery can be achieved by amphiphilic chitosan derivatives, such as lauroyl sulfated chitosan (LSC). LSC reversibly opens the tight junction and protects insulin from enzymatic degradation (Shelma and Sharma 2011). The functionalization of the chitosan nanoparticles with folic acid by ionotropic gelation method can augment the oral bioavailability of insulin (Agrawal et al. 2015). Recently, pH-responsive nanoparticle system comprising of chitosan and poly(γ-glutamic acid) is used for oral delivery of insulin. Chitosan has the property to adhere to mucosal surface and further opens the tight junction between epithelial cells (Fig. 6.2) (Sung et al. 2012).

Alginate, an anionic mucoadhesive polysaccharide consisting of β-D-mannuronopyranosyl and α-L-guluronopyranosyl units linked by (1,4)-O-glycosidic bonds, is widely used to prepare microparticles. Alginate exhibits the property of gel formation by ionically cross-linked multivalent cations whereby the drug is retained in the matrix. Calcium pectinate nanoparticles retard insulin release due to cross-linked alginate gel formation (Wong and Sumiran 2014). It has been seen that the alginate chitosan microspheres can load for oral delivery of insulin by different methods. It was observed that the highest loading efficiency occurs by solidification process in which the network formation reduces the porosity and leakage of insulin (Luo et al. 2016). Calcium phosphate nanoparticles, using vitamin B12-grafted chitosan and sodium alginate as the cationic and anionic polyelectrolyte, prepared by layer-by-layer approach are reported for oral delivery of insulin.

Poly-γ-glutamic acid (γ-PGA) is another biodegradable polymer which, in combination with chitosan, can be used for oral delivery of insulin. Due to their small size, they exhibit high loading efficiency compared to unmodified chitosan (Lin et al. 2007). γ-PGA conjugated covalently by diethylenetriaminepentaacetic acid (DTPA) could prevent enzymolysis and extend the residence time of the chitosan/γ-PGA-DTPA system for oral insulin delivery (Su et al. 2012). Another natural polymer used for nanostructured oral insulin delivery systems is starch. Starch acetate, when conjugated with polyethylene glycol for oral insulin delivery, exhibits increased mucoadhesiveness (Minimol et al. 2013). Hyaluronic acid is an anionic non-sulfated glycosaminoglycan natural polymer for insulin delivery. Insulin-loaded hyaluronic acid can be prepared by emul-

sion freeze-drying method in which the pH sensitivity of the nanoparticles protects insulin from acidic pH of the stomach. Similarly, vitamin B12 plays a significant role as a pH-sensitive element for enhancing oral bioavailability of insulin (Verma et al. 2016).

Synthetic Polymeric System

The use of synthetic polymeric carriers can be well controlled, and thereby biological property and drug release characteristics can be modified. Insulin can be entrapped in poly(lactide-co-glycolide) (PLGA) nanoparticles due to the hydrophobic interaction between insulin and PLGA by a solvent evaporation method (Minimol et al. 2013). The negative charge of PLGA nanoparticles exhibits less adhesiveness, but the bioavailability of PLGA–insulin nanoparticles can be enhanced by cationic modification (Zhang et al. 2012). Pluronic/poly(lactic acid) can also be used as vesicles for oral insulin delivery. Polymeric nanoparticles composed of biodegradable poly(Ɛ-caprolactone) and polycationic non-biodegradable acrylic polymer (Eudragit RS) are used for oral regular human insulin and insulin aspart delivery (Luo et al. 2016).

Oral insulin delivery continues to be a challenging risk due to enzymatic degradation from the gastrointestinal tract and the low level of absorption after oral administration. Polymeric hydrogels are considered to be one of the potential carriers for oral delivery of insulin. Among them, poly(D,L-lactic-co-glycolic acid)-polyethylene glycol (PLGA-PEG) nanoparticles act as a potential pH-responsive hydrogel, possessing the ability to protect insulin from gastrointestinal degradation as well as releasing insulin in the small intestine. PLGA is considered as an aliphatic biodegradable polyester carrier in drug delivery. The biodegradability of this carrier depends on the molecular weight and chemical compositions. Oral insulin delivery using PLGA-PEG nanoparticles has reduced the undesired interactions. The pH-sensitive polymer coating helps in the controlled release of the drug. The block copolymer of PLGA-PEG is prepared by the ring opening polymerization of the lactide and glycolide in the presence of PEG. Insulin is incorporated in the polymers by double emulsification method. However, the entrapment efficiency depends on several factors like copolymer concentration in organic solution, the volume of inner and outer aqueous phase, and homogenization speed and time (Hosseininasab et al. 2014).

6.3.6 Lipid-Based Nanocarriers

Liposomes

Liposomes are defined as biodegradable bilayered vesicles consisting of amphiphilic phospholipids. Their particle size ranges from nanometers to microns, and they are considered as carriers for various hydrophilic and hydrophobic compounds. The

hydrophilic interior phase helps in the entrapment of water-soluble insulin. The entrapped insulin is preserved from pH variation, enzymatic attack, and immune recognition. However, the antidiabetic property is dependent on lipid constituents, surface charge, and physical state of the phospholipid bilayer. Liposomes in combination with high-melting dipalmitoylphosphatidylcholine (DPPC) or negatively charged phosphatidylinositol show the remarkable effect on reducing blood glucose level.

The stability of the enzyme in the intestinal lumen and adhesion to intestinal epithelium can be enhanced by surface modification and ligand coating of the liposome. Coating with polyethylene glycol or mucin increases the stability of oral insulin delivery. Improved mucoadhesion can be achieved by chitosan coating of the liposome. Wheat germ agglutinin, tomato lectin, and *Ulex europaeus* agglutinin 1 when combined with N-glutaryl-phosphatidylethanolamine (N-glut-PE) can recognize and interact with sugar residues attached to either proteins or lipids in the cell membrane of the intestinal enterocytes. Oral delivery of these compounds in conjugation with insulin can maintain the glucose level up to 12 h. Moreover, biotin receptor-mediated endocytosis can be achieved by encapsulating insulin in biotin-modified liposome. The movement of liposomal insulin across the intestinal epithelium can be improved by incorporation of a permeation-enhancing agent (Mo et al. 2014). The destabilization against physiological bile salt and increased permeability in the gastrointestinal tract are achieved by formulating bilosome. Further increase in insulin bioavailability can be achieved by sodium glycocholate/insulin-loaded liposomes (Niu et al. 2012).

Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are colloidal carrier particles with a size range of 10–1000 nm. SLNs comprise a solid lipid core inflexible at room temperature imparting more stability. Insulin-loaded cetyl palmitate-based SLNs exhibit 24-h lasting hypoglycemic effect. The intestinal permeability of SLNs could be enhanced by coating with chitosan. In this case, a significant increase in bioavailability is seen when compared with uncoated SLN (Sarmento et al. 2007). Moreover, the improvement of bioavailability is seen with a modification of SLNs with wheat germ agglutinin. In a similar way, cetyl palmitate-based insulin nanoparticles produce a considerable hypoglycemic effect. The solid matrix of SLN protects insulin from chemical degradation in the intestinal tract and enhances the insulin absorption across the intestinal epithelium (Sarmento et al. 2007). A cationic insulin-loaded solid lipid nanoparticle is prepared by water-in-oil-in-water double emulsion technique (Hecq et al. 2016). Similarly, preparation of insulin-loaded lecithin-modified solid lipid nanoparticle is reported by a modified double dispersion method (Zhang et al. 2006).

Cell-penetrating peptides are promising ligands to enhance the property of SLN for penetrating the cell membrane. Thereby, cell-penetrating peptide improves the intestinal absorption of insulin. Cell-penetrating peptides are mainly classified as basic peptides, amphiphilic peptides, and hydrophobic peptides. Among them, octa-

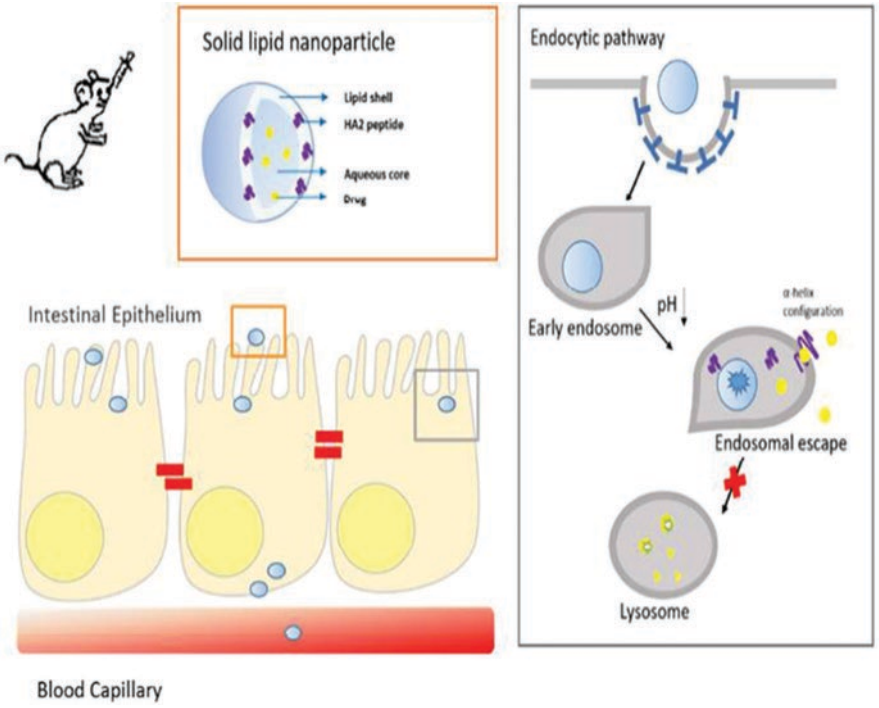

Fig. 6.3 Schematic representation of mechanism of transport of solid lipid nanoparticle encapsulated hemagglutinin-2 peptide and insulin. The solid lipid nanoparticle preserves the biological activity of insulin by endosomal escape. ("Reprinted with permission from (Xu et al. 2018). Copyright (2018) American Chemical Society")

arginine molecule enhances the penetration of SLN. The reaction is mediated by the attraction between positively charged arginine molecule and negatively charged cell membrane (Kaklotar et al. 2016). Interestingly, stearic acid-modified octaarginine-conjugated insulin SLNs show improved stability, enhanced uptake, and hypoglycemic effect (Hui-xia and Press 2012). Recently, SLNs consisting of an endosomal escape agent (hemagglutinin-2) loaded with insulin are reported for oral delivery of insulin (Fig. 6.3). Here, protonation of hemagglutinin-2 peptide induces a conformational change with respect to endosomal acidification. This system avoids lysosomal degradation and enhances transepithelial transport (Xu et al. 2018).

6.3.7 Insulin Bioconjugates

Greater results have been resulted from nanocarriers in drug delivery. However, the efficacy of cell-specific receptors that deliver drug to the targeted site is affected by the small size of the particle. In order to overcome the problem, the

protein is conjugated with targeting moiety to deliver the drug to the targeted site. A major challenge is that targeting efficiency can be hindered by the presence of mucous layer on epithelium. Highly specific targeting ligand needs to be used to solve the limitation.

An insulin bioconjugate comprises a transferrin molecule for oral delivery. Insulin bioconjugates are uptaken by the epithelial cells via receptor-mediated transcytosis, thereby increasing the permeability of insulin. A complexion hydrogel of insulin–transferrin conjugate stabilizes insulin from proteolytic enzymes and prevents the degradation of insulin. The mechanism of the conjugate is transferrin receptor-mediated transcytosis. The conjugate can be synthesized by the site-specific modification of insulin and modification of transferrin by a heterobifunctional cross-linker (Kaklotar et al. 2016). Increased stability is observed for the conjugated form of insulin. However, one of the limitations observed is that the transferrin-mediated transcytosis reaction is generally slow. This may be due to the polarity of transferrin receptor predominant in the basolateral surface of intestinal epithelial cells. The conjugation of insulin with transferrin takes place by a disulfide linkage. The transport of conjugated insulin could be further enhanced by conjugating with Brefeldin A (Kaklotar et al. 2016). It is important to note that transformation of the insulin–transferrin conjugate into a hydrogel further enhances the insulin stability from enzymatic degradation (Kavimandan et al. 2006; Shofner et al. 2010).

Recently, mucus-penetrating virus-inspired biomimetic nanoparticles having charge reversal property (P-octaarginine-phosphoserine nanoparticles) are prepared by densely coating PLGA nanoparticles with cationic octaarginine peptide and specific anionic phosphoserine. Intestinal alkaline phosphatase substrate was taken as the anionic group to form the viruslike particle. The charge reversal property helps in effective cellular internalization (Fig. 6.4) (Wu et al. 2018).

6.3.8 Iron Oxide Nanoparticles

Iron oxide nanoparticles, exhibiting unique physicochemical properties, are biodegradable and offer a promising approach for in vivo application. The laser ablation of a solid target immersed in a liquid environment is mainly used for the fabrication of the nanostructured materials (Boyd 1998). Noble metal nanoparticles with the bare surface can be prepared by a physical approach. Noble metal nanocomposite for oral delivery of insulin is produced by pulsed laser ablation technique. This method is chemical-free and provides an alternative to the traditional painful method of injection.

Chitosan can easily disperse metal oxides (Kaushik et al. 2008). This property makes it a good dispersant for the iron oxide nanoparticles. Iron oxide nanoparticles exhibit a hydrophilic surface, whereas the conjugation of chitosan with fatty acid as hydrophobic media enhances the absorption of insulin (Kebede et al. 2013). Impurity-free controlled preparation of iron oxide nanoparticle is possible by eco-

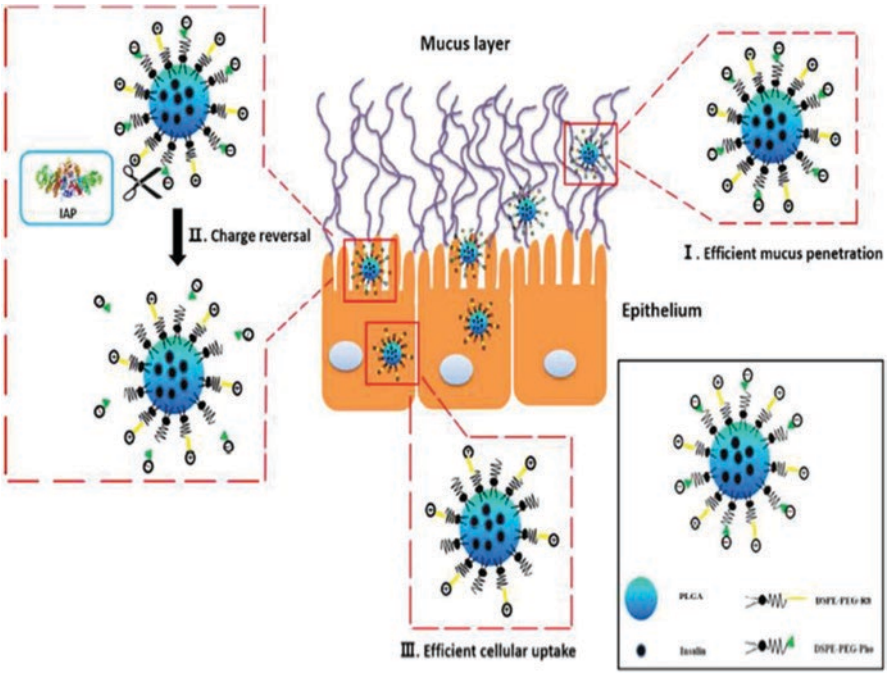

Fig. 6.4 Schematic representation of the mechanism of transport of P-octaarginine-phosphoserine nanoparticles. The prepared nanoparticles showing charge reversal property result in effective cellular internalization. ("Reprinted with permission from (Wu et al. 2018). Copyright (2018) American Chemical Society.")

friendly laser ablation technique in ultrapure water and chitosan solution. This delivery system produced reduction in glucose level to 50 % in vivo (Kebede et al. 2013).

6.3.9 Chondroitin Sulfate-Capped Gold Nanoparticles

The synthetic approach used for the synthesis of gold nanoparticles (AuNP) determines the size, shape, and surface functionality (Yeh et al. 2012). Various substances are used for the synthesis of AuNP. Among them, bifunctional compounds elicit their own functionality and reducing property (Kim et al. 2013). Chondroitin sulfate is used as a reducing agent for the synthesis of AuNP. It is a sulfated glycosaminoglycan made of N-acetylgalactosamine and glucuronic acid. It is mainly present in the extracellular matrix and helps in maintaining the structure of the tissue. The negative charge of chondroitin sulfate makes it interact with the proteins in the extracellular matrix. It is used in drug delivery system because of its biocompatibility and ability to interact with biological components. In the oral delivery of insulin, chondroitin sulfate is mixed with AuNP as a reducing and stabilizing agent (Cho et al. 2014).

6.3.10 Insulin-Entrapped Liquid Crystalline Nanoparticles

Liquid crystalline nanoparticles (LCNPs) are well-defined thermodynamically stable self-assembled lipid structures which are formed when these lipids are exposed to the polar water phase. Insulin-loaded LCNPs are prepared by a hydrotrope method where a specific concentration of lipid, solvent, and surfactant is used. Here, insulin is protected in the hydrophilic channel during the generation of LCNP. Lecithin is added to the formulation to impart a negative charge. Thereby, it increases the entrapment between the negatively charged LCNP and positively charged insulin molecules. The addition of surfactants offers advantages like prevention of aggregation of nanoparticles by steric stabilization, maintaining the nanosize, and also increasing the miscibility of the hydrophilic drug in hydrophobic environment further leading to improved absorption. Insulin, being a sensitive molecule, should preserve the conformational stability to maintain the biological activity. Therefore, conformation studies are needed with different formulation ingredients during initial screening (Fig. 6.5) (Agrawal et al. 2017).

Insulin-loaded LCNPs provide a sustained release of insulin. The in vitro release rate was observed to be lower during initial hours followed by 90% release within 24 h. High cell uptake is observed with fluorescein isothiocyanate-labeled insulin-loaded LCNP than free fluorescein isothiocyanate insulin. However, physical instability in suspension form was a major problem which is avoided by adopting freeze-drying. But freeze-drying produces a stress that destabilizes the colloidal suspension and induces aggregation and irreversible fusion of nanoparticles. The induction of stress can be avoided with the use of cryoprotectants such as mannitol with the formation of a glassy matrix. Other cryoprotectants result in a poor matrix formation. The reported LCNPs show high cellular uptake, higher stability, and sustained glucose-lowering effect. Ease of development and cost-effectiveness are the major merits associated with LCNPs (Agrawal et al. 2017).

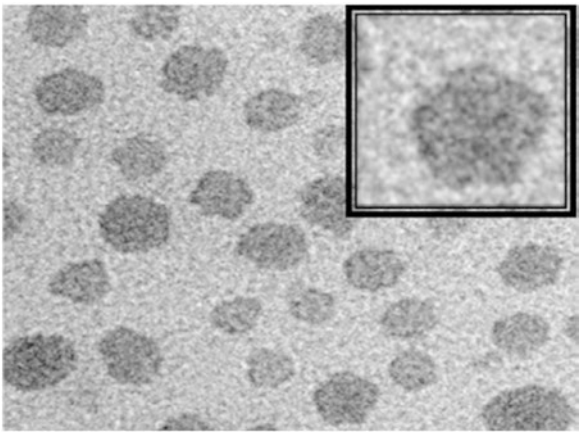

Fig. 6.5 Surface morphology of liquid crystalline nanoparticle by transmission electron microscopy. The image reveals that the liquid crystalline nanoparticle has hexagonal structure. ("Reprinted with permission from (Agrawal et al. 2017). Copyright (2017) American Chemical Society")

6.3.11 Silica Nanoparticles

Mesoporous silica nanoparticles provide a definite arrangement of pores and channels with different geometries. The large surface area, high biocompatibility, uniform-sized pore, easy chemical functionalization, and easiness of synthesis are some of the characteristics that make them important as drug delivery vehicles. It is based on the fact that silanol group attached to the mesoporous walls absorbs drug carriers of interest (Siavashani et al. 2013). Coating of nanoparticle with selective mucoadhesive polymer results in an improved oral bioavailability of therapeutic proteins. Studies are reported on the interaction of insulin and silica nanoparticles with different mucoadhesive polymers such as chitosan, polyethylene glycol, and sodium alginate (Andreani et al. 2014). Activated nano silica is formed by 3-chloropropyltrimethoxysilane with 4-hydroxybenzoic acid. The interaction results from the replacement of chlorine group with the organic functional group (hydroxyl group), resulting in pH-sensitive response. Here, insulin is entrapped in the matrix. At pH 7.4, deprotonation of the silanol groups takes place leading to increased drug release rate. The system is suitable for colon-specific insulin delivery (Mahkam 2011). The functionalization of the surface of porous silica nanoparticles with cell-penetrating peptides could be used in the oral delivery of insulin, where the cell-penetrating peptide increases the permeability across the intestinal cells.

6.3.12 pH-Sensitive Nanostructured Polyelectrolyte Microparticles

Proteins could be incorporated into polyelectrolyte microparticles by stepwise deposition of oppositely charged polyelectrolyte. Proteins can be immobilized by two methods: one by the inclusion of protein into ready-made polyelectrolyte microparticles and another by the formation of microparticles in a matrix containing protein. Protein immobilization approach is simpler and promising method for oral insulin delivery system. The main advantage of this method is the simplicity of the procedure and instrumentation. The reactions are mainly done at room temperature. Moreover, protein-encapsulated polyelectrolyte microparticles are sensitive to pH changes. Various biopolyelectrolytes such as chitosan sulfate and dextran sulfate can be adsorbed onto a protein-containing matrix. Thereby, insulin containing microparticles can be fabricated which is protected from the gastric environment and releases it in the small intestine. These characteristics make pH-sensitive nanostructured polyelectrolyte microparticles a promising approach to the oral delivery of insulin (Balabushevich et al. 2010). Formation of

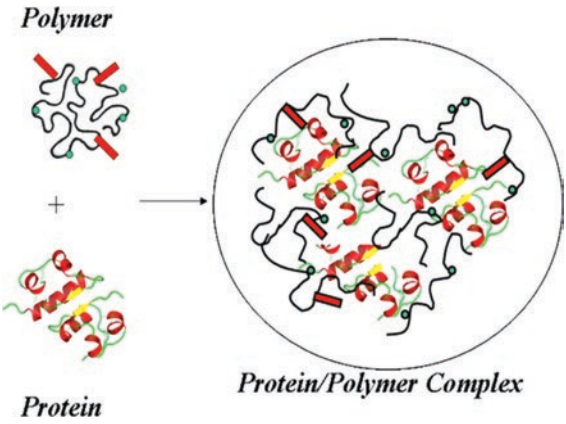

Fig. 6.6 Schematic representation of the formation of a protein/polymer complex. The copolymers combine with polymer molecules to form nanoaggregates. ("Reprinted with permission from (Mariano et al. 2013). Copyright (2013) American Chemical Society")

nanoaggregates based on interaction with copolymers and protein molecule can serve as an ideal material for oral insulin delivery (Fig. 6.6) (Mariano et al. 2013).

6.3.13 Multilayer Nanoparticles

Multilayered nanoparticles protect insulin from enzymatic degradation in addition to increasing insulin absorption. A complex multilayered nanoparticle is incorporated in hydrogel comprising of alginate, dextran sulfate, and poloxamer. Incorporation of insulin into a hydrogel gel can be stabilized by chitosan and albumin coating. Poloxamers possess the property to enhance drug solubility and transport in the intestine. Fortunately, these steps do not affect the in vitro bioactivity of insulin. Smaller nanoparticles can be prepared by decreasing poloxamer and albumin concentration. Improved insulin entrapment can be achieved by increasing polymer concentration (Ahmad et al. 2012).

6.3.14 Electrospun Nanofibers

Electrospinning is the production of nanofibers by applying a high-voltage direct current to polymer solution. Different types of polymers were electrospunned to produce different fibers. The great surface area of nanofibers helps in improving the bioavailability of poorly soluble drugs (Ignatious et al. 2010). Recently, a sustained and controlled release active antidiabetic oral formulation was developed using electrospun nanofibers. Electrospun composite nanofiber transmucosal patch was prepared using polyvinyl alcohol and sodium alginate. Insulin was incorporated by active loading. The formulation exhibits desired therapeutic effect (Ignatious et al.

2010). The fibers tend to be instable without cross-linking. It has been reported that chitosan nanofibers were used in oral insulin delivery.

6.4 Nanostructured Insulin Delivery Systems Under Pipeline

Pharmaceutical companies are still trying to develop a suitable system for oral insulin delivery. Many of the products are under developmental phase and some under clinical trials. The main aim is to avoid the gastrointestinal degradation and promote intestinal uptake which can be achieved by protease inhibitor and absorption enhancer (Fonte et al. 2015). Chitosan-4-thiobutylamidine tablets, wherein an enzyme inhibitor is covalently linked to insulin for bypassing the release in GIT, are developed (Krauland et al. 2004). CODES™ tablets comprising insulin, lactulose, meglumine, polyethylene oxide, citric acid, and sodium glycocholate are developed. Lactulose promotes drug release in the colon, citric acid as pH adjuster, meglumine as insulin solubilizer, sodium glycocholate as absorption enhancer, and finally polyethylene glycol forms a gel barrier for sustained release of insulin (Katsuma et al. 2006).

Capsulin™ (Diabetology Ltd, UK), an enteric-coated capsule filled with a mixture of insulin, an absorption enhancer, and a solubilizer, is under phase II clinical trials. A similar type of enteric-coated capsule is produced by Oramed (Jerusalem, Israel) as ORMD-0801. BOWS Pharmaceuticals AG (Switzerland) developed ORA2 which is a capsule containing insulin in dextran matrix. Eligen capsule by Emisphere Technologies (New Jersey, United States) and NN1952 by Novo Nordisk (Bagsvaerd, Denmark) were canceled after phase II trials. Long-acting insulin analog tablet developed by Novo Nordisk (Bagsvaerd, Denmark) is under phase I development. Insulin modified with PEG is under development as IN-105 by Biocon (Bangalore, India). Liposomal insulin HDV-I as a hepatic-directed vesicles in orally administered forms has completed phase III trials (Zijlstra et al. 2014).

Access Pharmaceuticals, Inc. (Dallas, TX, USA) developed CobOral™ technology, a polymer-based delivery system with vitamin B12 uptake mechanisms in the intestine to increase oral delivery of insulin. Upon oral administration, vitamin B12 attached to nanoparticles' surface interacts with haptocorrin in the stomach, and further migration and dissociation of the complex to duodenum take place. The intrinsic factor released in stomach binds to vitamin B12, resulting in the formation of a complex, which then interacts with the intrinsic factor receptor in the ileum. Thereby, the conjugated nanoparticles with vitamin B12 reach the bloodstream by an endocytotic process. A novel nanoparticle delivery system in which a core of neutral γ-polyglutamic acid is coated with chitosan was developed. The chitosan helps in the mucoadhesion of the particle and γ-polyglutamic acid for nanoparticle solubilization. Only a few results were promising, though limited in the extent of performance, in the oral delivery of insulin. Further work needs to be carried out for the development of a successful oral insulin delivery system (Zijlstra et al. 2014).

6.5 Conclusion

Oral insulin delivery results in increased patient compliance due to its ease and simplicity. Nanotechnology makes a promising platform for the delivery of insulin through nanocarriers. Nanomedicine has resulted in enhanced delivery of insulin to the absorption sites. The oral insulin can be delivered by various transcellular and paracellular mechanisms. Surface functionalization of the nanoparticles or various combination therapies has resulted in increased delivery of insulin to systemic circulation as well as enhanced stability of insulin in gastrointestinal tract. Targeted delivery of insulin takes place by microfold cells or by receptor-mediated endocytosis. Conjugation of insulin with various nanostructured carriers has resulted in the improved bioavailability of oral insulin. However, high doses of insulin to intestinal tissue results in mitogenic changes. Therefore, a nanostructured delivery system should be developed such that they deliver a low dose of insulin to the site.

Subcutaneous administration of insulin results in a low availability of insulin to the liver. Thus, oral administration results in an enhanced amount of insulin in liver than systemic circulation. Therefore, oral insulin delivery is a promising approach for the treatment of diabetes. Search is still undergoing for an efficient oral insulin system. Mostly, oral insulin delivery systems are formulated such that they react with intestinal epithelium. Therefore, it is important to consider that that the system does not cause any toxicity to the tissue. This can be accomplished by various biodegradable nanocarriers. Further studies are needed to test the efficacy and safety of the nanomedicines of insulin. Overall, nanostructured oral insulin delivery is a promising approach to enhance the therapeutic efficacy of insulin.

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Chapter 7
Electrospun Nano-architectures for Tissue Engineering and Regenerative Medicine

Vandana Joshi, Chandra Mohan Srivastava, Anek Pal Gupta, and Monika Vats

Contents

Abstract Skin consists of three layers, namely, epidermis, dermis, and hypodermis. In case of partial injury to the epidermis layer, the body has the ability to heal itself naturally, but in case of deep dermal injuries, skin substitutes are required. This skin transplantation can be done by using either allograft or autograft or xenograft. However, these techniques are associated with drawbacks like high cost, limited availability, and disease transmission. In order to mitigate these challenges, tissue-engineered skin grafts can be used. Nowadays, researchers are trying to engineer artificial organs which will help patients facing organ failure and would

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end the hassle of finding a suitable donor. This rapidly emerging field of science is known as regenerative medicine. Regenerative medicine involves repairing or engineering human tissues and organs by culturing normal cells or stem cells on scaffolds. To ensure the growth of cells, these scaffolds must be porous, should have good water-holding capacity, and should allow easy permeation of gases and metabolites. Nanofibers due to their unique properties like large surface area, high porosity, and increased mechanical strength are considered as ideal material for scaffold preparation. It has been found that nanofibers help in promoting adherence, growth, and proliferation of seeded cells and successful development of tissue-engineered constructs. Electrospinning is a cost-effective, simple, and versatile method which can be used for fabrication of a variety of nanofibers at a large scale. By changing various parameters like voltage, concentration of solution, tip to collector distance, feed rate, speed of collector drum, and viscosity, the orientation and diameter of nanofibers can be fine-tuned to match the desired end applications. Orientation of nanofibers, porosity, pore size, and nanophase surface roughness are some of the factors that have a great influence on cell growth. It has been observed that smaller size of fibers than the cell size facilitates the orientation of the cells around the fiber. Pore size has also been found to affect the cell morphology. On decreasing the pore size of randomly oriented nanofibrous membrane, the cell morphology changes from spherical to elongated, whereas in the case of aligned fiber membrane, on decreasing the pore area, the cell remains in elongated state and is found to spread along the direction of alignment of fiber. Although significant amount of work has been carried out to study the role of 'nanofibers diameter' on the adherence, growth, and proliferation. The effect of fiber orientation and pore size on cell adhesion is still not fully explored. In this chapter, we review (1) general properties of nanofibers and biopolymers, (2) electrospinning process and its types, (3) parameters which affect the electrospinning process, (4) applications of the electrospun nanofibers in the field of regenerative medicine, and (5) existing regenerative medicine products in the market. The major applications discussed are tissue engineering and drug delivery, and a detailed discussion regarding regeneration of different types of tissues has been carried out. A comprehensive list of electrospun and co-spun biopolymers along with their spinning condition and potential applications has been tabulated by thorough literature analysis. This review aims to identify the research gap in this field and to highlight the future prospects of this efficient technology in the field of medicine.

Keywords Electrospinning · Biopolymers · Regenerative medicine · Nanofibers · Tissue engineering · Coaxial · Melt spinning · Drug delivery · Skin substitute · Scaffold

7.1 Introduction

Fibers having diameter less than or equal to 100 nm are called nanofibers. These nanofibers due to their remarkable properties like high surface area-to-volume ratio, flexibility, high porosity, and appreciable mechanical strength have tremendous applications. Electrospinning is the most popular technique for generation of nanofibers. This chapter explains the fabrication process and the effect of various parameters on the formed nanofiber along with various types of electrospinning processes as well as recent innovations in electrospinning machines. Applications of biopolymer nanofibers in the field of tissue engineering and regenerative medicine have been discussed in detail. This chapter also summarizes the various products available in the market for tissue engineering and regenerative medicines applications.

7.1.1 Nanofibers

Properties such as surface-to-volume ratio, flexibility, mechanical strength, etc. drastically change when dimension of a material transforms from microns to nanometers, resulting in improved quantum efficiency, better surface energy, and higher surface reactivity as well as superparamagnetism and increased thermal and electrical conductivity (Bean and Livingston 1959). These properties make polymer nanofibers suitable for various applications like air and water filtration, drug delivery, tissue engineering, and wound dressing. Various nanofibers of polymer like polyurethane (Kim et al. 2009), gelatin (Jegal et al. 2011), and collagen (Rho et al. 2006) have been fabricated and studied for a number of medical applications. These nanofibers can be prepared by various techniques such as drawing (Ondarcuhu and Joachim 1998), template synthesis (Feng et al. 2002; Martin 1996), phase separation (Ma and Zhang 1999), self-assembly (Liu et al. 1999; Whitesides and Grzybowski 2002), and electrospinning (Deitzel et al. 2001).

7.1.2 Biopolymers

Natural occurring polymers generally known as biopolymers. Biomaterials such as crustacean shells, wood, mushrooms, etc. have been used to make biopolymers. Properties like sustainability, industrial efficiency, and renewable nature make their useful application in medicine and other fields. Biopolymers are renewable (Kaplan 1998), biocompatible, and biodegradable and also show antibacterial activity (Rinaudo 2006; Kumar 2000; Subbiah et al. 2005; Berger et al. 2004; Chirkov 2002; Dodane and Vilivalam 1998; Vartiainen et al. 2004). Electrospun biopolymer fibrous mats have also been used for making protective clothing and nanocomposites (Huang et al. 2003).

7.2 Electrospinning

Electrospinning or electrostatic spinning technique has been used for the preparation of polymeric fibers having diameter in range of submicron to nanometer (natural or synthetic). The electrospun fibers are drawn out from the polymer solution or melt using electric force. The electrospinning techniques is quite similar to electrospraying or the conventional dry spinning. The consistency of the fiber produced in the submicron range is an advantage over the other mechanical spinning techniques, which is otherwise difficult to obtain. The process was discovered by Lord Rayleigh; however, first patents for electrospinning were published by J.F. Cooley and W.J. Morton in 1902. Anton Formhals work leads to commercialization of its usage; he used a voltage of 57 kV for electrospinning of cellulose acetate for fabrication of textile yarns (Formhals 1934; Formhals 1939; Formhals 1940; Formhals 1943; Formhals 1944). The technique produces nanofibrous mats with controllable pore structure and improved porosity and surface area. The technique is cost-effective, moderately easy, and replicable for producing nanofibers (Jayaram et al. 2004).

7.2.1 Types of Electrospinning

Depending on state of spinning materials (solution or melt) and needle type, there are three most important electrospinning machines in use, namely, solution electrospinning, coaxial spinning, and melt spinning.

Solution Electrospinning

Electrospinning apparatus as shown in Fig. 7.1 is composed of three basic components: high-voltage supplier, pipette or capillary tube with a needle, and a collector screen. The capillary is filled with polymer solution or polymer melt whose nanofibers are desired. One end of the power supply is connected to the capillary tube, whereas the other end is connected to the collector screen. There is short distance between the capillary tube and the collector screen. Some examples of materials used as collector are rotating drums (Wannatong et al. 2004; Kim et al. 2004; Chew et al. 2005), copper plates (Schiffman and Schauer 2007a, b), and aluminum foil (Li et al. 2006; Ji et al. 2006; Gopal et al. 2007).

An advance pump forces the polymer solution from the syringe to the needle. The polymer solution at the end of capillary is held by surface tension. The end of the capillary is subjected to an electric field which induces electric charge on liquid surface. Electric field increases with increase in voltage which builds up a force on the pendant drop. This force opposes the force of surface tension. With increase in electrostatic force, the fluid at the tip of the capillary elongates to a conical shape known as Taylor cone (Taylor and Dyke 1969). At critical value of electric field, the

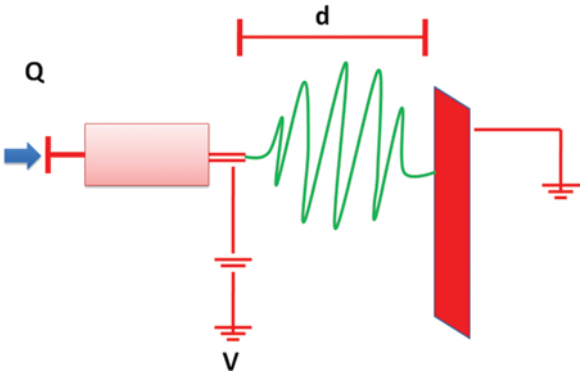

Fig. 7.1 Basic electrospinning setup. The polymer to be electrospun is pushed out of the capillary tube into the needle at the flow rate "Q." A voltage "V" is applied to the tip of needle, and another end of the power supply is connected to the collector plate placed at a distance "d." Due to electrostatic force, the fiber travels through the air with bending and whipping. During this flight, the solvent evaporates, while the fiber elongates, and a nonwoven mat of randomly oriented nanofibers is deposited on the collector screen

electrostatic force overcomes surface tension, and a continuous charged jet of solution is ejected from the surface of cone bending and whipping in every direction. The solution jet is unstable during the flight to collector and gets elongated. The jet thins and the solvent evaporates, leaving behind nonwoven mat of randomly oriented nanofibers on the collector screen (Haghi and Akbari 2007; Purwar et al. 2016; Zhang et al. 2005a).

Coaxial Electrospinning

Coaxial electrospinning enables the formation of core-sheath-structured fibers in submicron range. Just like electrospinning, coaxial electrospinning uses electric field acting on polymer solutions which results in stretching and thinning of polymer jets. Figure 7.2 illustrates the experimental setup used in coaxial electrospinning. The apparatus is similar to that used for solution electrospinning with slight modifications in the spinneret. A smaller capillary (inner) is inserted that concentrically fits inside the bigger (outer) capillary to make coaxial configuration. Two different polymer solutions for sheath and core materials are stored separately in a reservoir.

The reservoir containing sheath solution is connected to the outer needle, whereas the one containing core solution is connected to inner needle. Both of these solutions are ejected simultaneously. Due to electric field, charge accumulates on the surface of the sheath liquid coming out of the outer coaxial capillary (Greiner et al. 2006). Elongation and stretching in the pendant droplet of sheath solution occur due to repulsion of like charges, and it forms a conical shape. When the voltage reaches

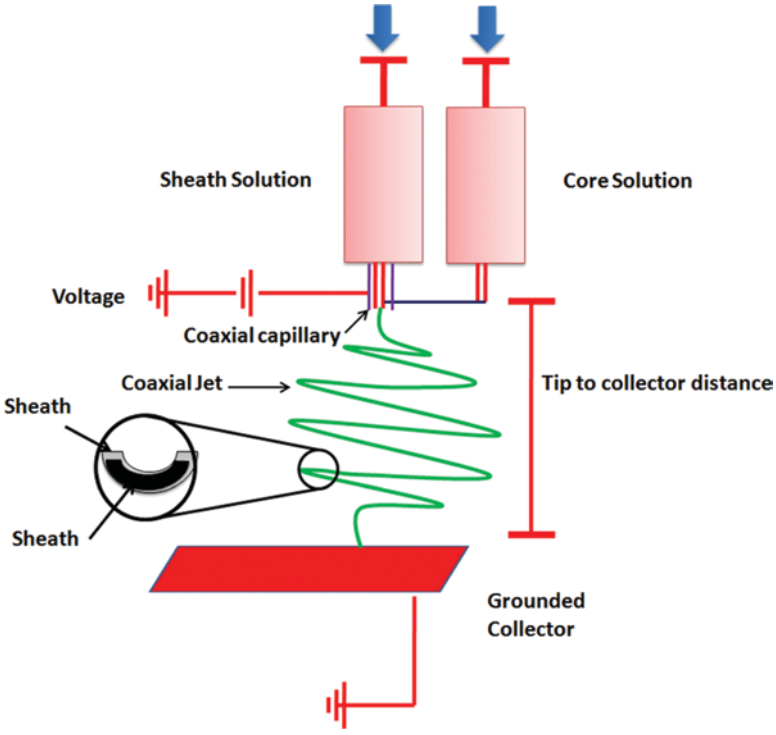

Fig. 7.2 Basic experimental setup for coaxial electrospinning. The apparatus is similar to that used for solution electrospinning with slight modifications in the spinneret. A smaller capillary is placed inside a bigger capillary. Both of these are connected to reservoirs containing different polymer solutions due to which the solution coming out of the smaller capillary (core) is covered by a layer of solution that is being ejected from bigger capillary which leads to the formation of core-sheath nanofibers

a threshold value, a fine jet is ejected which consists of core material enclosed within the sheath material. This compound jet undergoes bending instability just like in conventional electrospinning. During the flight, liquid jet is whipped and stretched, and both the solvents get evaporated which leads to the formation of solid-state core-sheath fibers. Using coaxial electrospinning different properties of polymers can be combined into one fiber. Coaxial electrospinning was first demonstrated by Sun et al. (2003) by using two different polymers for sheath and core.

Melt Electrospinning

Melt electrospinning is a fiber fabrication technique in which electric potential is applied on polymer melt to generate nanofibers. Contrary to the more famous solution electrospinning, melt electrospinning does not require a solvent for dissolution of polymer. In melt electrospinning, we prepare nanofibers from molten polymer.

The instrument used for melt electrospinning is similar to that of solution electrospinning except that it contains a heating assembly and a temperature controller. The heating assembly melts the polymer to a suitable viscosity which on applying electric potential forms elongated electrified jets. The heating assembly can provide heat by various sources such as heating gun, heating element, laser beam, etc.

Molecular weight is a crucial factor in melt spinning. Only those polymers whose molecular weight lies in an optimum range (40,000–80,000 g/mol (Brown et al. 2011)) can be electrospun easily. Polymers with low molecular weight result in broken and poor quality of fibers, whereas those with high molecular weight due to their high viscosity have difficulty in flowing through the spinneret.

The bending instabilities observed during flight of the jet are much weaker in melt electrospinning (Zhou et al. 2006) and sometimes completely absent (Shin et al. 2001). This is due to high viscosity of polymer melt (Taylor and Dyke 1969). Another reason for occurrence of bending instability in solution electrospinning is presence of high surface charge density, but as majority of polymers are electrical insulators, there is no such instability observed in melt spinning. These bending instabilities help to reduce the jet diameter to nanometers, and their suppression prevents the stretching of the electrified jet, and fibers with diameter in range of micrometer are obtained. To increase these bending instabilities, higher voltage is required (Karchin et al. 2011). In addition, the whipping motion becomes vigorous at lower flow rates.

7.2.2 Parameters that Affect Electrospinning

Electrospinning is governed by various parameters. These parameters are categorized into the following categories:

(a) Solution parameters: It includes conductivity, viscosity, surface tension, etc.
(b) Process parameters: It includes applied electric field, flow rate, tip to collector distance, and needle diameter.
(c) Ambient parameters: It includes temperature and humidity.

Some of the parameters are discussed below:

(i) Applied Voltage.

An increase in applied electric potential increases the charge in a jet segment which causes an increase in electrostatic force as well as coulombic repulsion. These two forces act in a contradicting manner. Due to increased coulombic force, there is higher stretching of the Taylor cone due to charge repulsion within the jet which results in reduced fiber diameter and also accelerated evaporation of the solvent (Larrondo and Manley 1981a, b, c; Sill and von Recum 2008). On the other hand, increase in electrostatic force increases the amount of fluid that is being ejected which results in fibers of larger diameter (Zhang et al. 2005b; Purwar et al. 2016).

(ii) Solution Flow Rate.

To obtain uniform beadless nanofibers, a critical flow rate of polymer solution is necessary during the electrospinning process. This critical value varies from polymer to polymer. A high flow rate results in large fiber diameter. It also causes the formation of beads as the nanofiber doesn't get sufficient drying time during its flight from needle tip to collector (Megelski et al. 2002). A minimum flow rate is preferred to give sufficient time to the deposited fiber to settle down before ejection of the next jet (Zeleny 1935). High flow rates sometimes result in ribbon like defects (Megelski et al. 2002) and unspun droplets (Zargham et al. 2012). Due to increased flow rate, there is low stretching of jet and non-evaporation of solvent during the flight time which is the reason for increased fiber diameter, formation of beads and ribbon like structure (Li and Wang 2013). Influence of gravitational force is the reason for presence of unspun droplets (Zargham et al. 2012).

(iii) Tip to Collector Distance.

The tip to collector distance should be such that it allows the evaporation of solvent from the jet. There are two contradictory theories which relate the fiber diameter with tip to collector distance. According to one theory with the increase in tip to collector distance, the time available for evaporation increases which results in fibers with smaller diameter, whereas the other theory states that with increase in distance, the electrostatic field on the liquid droplet becomes weaker which results in formation of thick fibers.

(iv) Concentration and Viscosity.

Solution of optimum concentration is required for electrospinning as at low concentration due to insufficient molecular entanglement between polymer chains, the fiber breaks before reaching to collector (Haider et al. 2013; Pillay et al. 2013). This fragmentation is due to the force exerted by applied voltage and surface tension that results in formation of beaded nanofibers. At high concentration, the viscosity of polymeric solution increases which increase entanglement of polymeric chains which overcomes the surface tension and leads to formation of uniform beadless electrospun nanofibers. Very high concentration hinders the solution flow through the nozzle tip (due to increased viscosity, the needle tip may get clogged) and is responsible for formation of beaded nanofibers (Haider et al. 2013; Sukigara et al. 2003).

(v) Surface Tension.

It has been observed that high surface tension of a polymer solution makes the jet unstable and leads to formation of sprayed droplets (Hohman et al. 2001). By diminishing the surface tension of the solution, it is possible to obtain fibers without beads.

(vi) Conductivity.

Conductivity of solution is an important factor as charge on the surface of droplet is essential for formation of Taylor cone. Without the surface charge, the applied electrostatic force won't be enough to generate a Taylor cone and start the electrospinning process. It has been observed that diameter of elec-

trospun fibers decreases significantly with increase in conductivity of solution; however, highly conductive solutions are very unstable in strong electric field which causes high bending instability and broader fiber diameter (Hayati et al. 1987).

(vii) Temperature and Humidity.

Increase in temperature decreases the viscosity, and hence the diameter of fiber increases (Mit-uppatham et al. 2004). When humidity is very low, the solution dries very rapidly, and the needle tip gets clogged (Baumgarten 1971). High humidity assists discharge of electrospun fibers and fibers takes more time for drying (Li and Xia 2004a, b; Li et al. 2005a, b; Yuan et al. 2004; Wannatong et al. 2004; Zuo et al. 2005; Kim et al. 2005).

(viii) Drum Speed.

It is reported that speed of drum plays very important role on diameter and orientation of the nanofibers. By increasing the speed of drum, the diameter of nanofibers decreases, and alignment of fibers in direction of applied voltage takes place (Wannatong et al. 2004).

(ix) Collector Type.

Types of collector also have marked influence on the morphology of nanofibers. On plate-type collector, we generally get randomly oriented nanofibers, whereas we can prepare aligned nanofibrous mat using drum-type collector (Chew et al. 2005).

In a nutshell, all the parameters are interdependent. These parameter strongly depend on the structure of the polymer that is being electrospun.

7.3 Applications of Electrospun Nanofibers

When unusual properties of nanoparticles are combined with inherent properties of biopolymers, it leads to materials with a wide variety of applications. The nanobiofibers have been used as air filters as well as templates (Bognitzki et al. 2000) & scaffolds for tissue engineering (Meng et al. 2007) and regenerative medicines (Dilamian et al. 2013). Nanofibre mats have also been used for protective clothing (Gibson et al. 2001). An emerging application of nanofibers is in the medical field, where excellent research is going on. The nanofibers have established themselves as excellent wound dressing material owing to their ability to produce a moist (hydrated) atmosphere around the wound High porosity, surface area, appropriate water vapor transmission rate, and interconnected pattern resembling extracellular matrices further stimulates healing of chronic wound (Liu et al. 2017).

Chitosan, silk, and poly(D,L-lactide-co-glycolide) have intrinsic properties like biodegradability, biocompatibility, and non-toxicity due to which they have been used as frameworks in field of tissue engineering (Meng et al. 2010). The nanofibers are usually incorporated with antibacterial active particles to enhance properties. Due to their small size, nanofibers show improved quantum efficiency, better surface

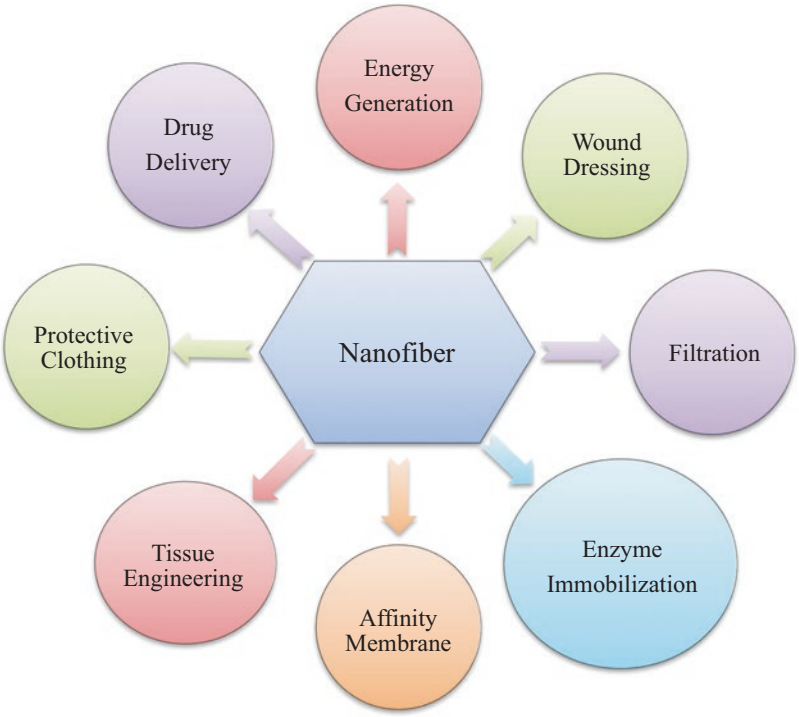

Fig. 7.3 Applications of electrospun nanofibers in different sectors

energy, and higher surface reactivity which makes them suitable for a wide range of applications.

The conventional materials used for wound dressing, designing tissue scaffolds, and drug delivery are swiftly being replaced by nano-biopolymer. It is evident that nanofibers of biopolymers have tremendous applications, some of which are shown in Fig. 7.3.

The electrospinning method is mostly applied in biomedical field (Burger et al. 2006) which are summarized below.

7.3.1 Scaffolds for Tissue Engineering

Scaffold acts as template for cells in tissue engineering, and their success lies in its ability to promote adherence, growth, and proliferation of cells. Nanofibrous scaffolds have high surface area and porosity that affect the cellular compatibility. Different types of synthetic as well as biological polymers have been successfully used as scaffolds for regeneration of tissue.

Biopolymers exhibit better biocompatibility and low immunogenicity due to which they have capacity for binding cells. Biopolymers carry particular tripeptide sequences such as R(Arg)-G(Gly)-D(Asp) especially in non-mulberry silk fibroin proteins and hence used for producing these scaffolds. These scaffolds must be designed in a way that they secrete own natural extracellular matrix and thus can be used for the treatment and replacement of injured tissues. It has been reported that human cells get attach or organize around fibers with diameter less than that of the cells; therefore, fibers with large diameter do not have morphological properties of the native fibrils (Laurencin et al. 1999), indicating nanofibers may serve the purpose.

A large number of scaffolds of polymers such as collagen, chitin, and chitosan have been used for tissue engineering such as heart valves (Du et al. 2018), cartilage (Aliakbarshirazi and Talebian 2017), bones (Song et al. 2008), dermal tissue (Rnjak-Kovacina et al. 2012), muscle cells (Choi et al. 2008), etc. Collagen being the most abundant protein in human body and the main structural component of extracellular matrix (Matthews et al. (2002) is extensively used in tissue engineering. The highly porous electrospun fibrous mats of poly(D,L-lactide-co-glycolide) with large surface area have been used for making scaffolds which promote cell growth and proliferation.

Tissue engineering is a vast field and has various subdisciplines. The use of nanofibers in some of the subdisciplines has been reviewed below.

Bone Regeneration

Bone matrix is a composite which is made up of type 1 collagen fibers and nanohydroxyapatite. It makes up 90% of bone tissue volume (Ross and Pawlina 2006).

Various synthetic and natural polymers have been electrospun with hydroxyapatite to create artificial extracellular matrix for bone regeneration. Zhang et al. (2008) fabricated hydroxyapatite/chitosan electrospun nanofibrous scaffolds with an average fiber diameter of 214 ± 25 nm. The presence of hydroxyapatite nanoparticles in chitosan scaffolds helped in proliferation and mineral deposition of human fetal osteoblast. The cellular activities on the scaffolds are affected by the pore size, porosity, and degree of pore interconnectivity of the nanofibers. The average pore size increases with increase in fiber diameter. Scaffolds with large pore size are essential for the growth of osteoblast cells and for bone formation (Yang et al. 2001; Whang et al. 1999). Song et al. (2008) fabricated electrospun biodegradable gelatin/siloxane nanofibers which showed enhanced osteoblastic activity as compared to nanofibers of pure gelatin.

Cartilage Regeneration

Nanofibrous scaffolds resemble the native cartilage extracellular matrix which makes them suitable for tissue engineering applications. The nanofibers spun from synthetic as well as natural biopolymer support the chondrogenic differentiation of

stem cells (Subramanian et al. 2005; Shin et al. 2006; Alves et al. 2010). Aliakbarshirazi and Talebian (2017) have successfully electrospun gelatin nanofibers for cartilage tissue engineering.

Tendon Regeneration

Ligament and tendons can be repaired using electrospun nanofibrous scaffolds as an artificial extracellular matrix since they mimic the collagen fiber bundles present in native tissues. Taylor et al. (2010) developed poly(D,L-lactide-co-glycolide) nanofibrous scaffolds via electrospinning. These scaffolds were used for healing of a torn rotator cuff. Scaffolds made of aligned nanofibers resemble the anisotropic structure of closely packed collagen fibers present in a tendon.

Cardiac Tissue Engineering

Diseased peripheral and coronary arteries can be treated with the help of bypass surgery, but due to unavailability of suitable vein, prosthetic vascular graft has to be used in some cases. These prosthetics although suitable in large-bore applications do not perform well for small-diameter applications due to difficulty with thrombosis, graft occlusion, and infection (Swain et al. 2004; Grego et al. 2003; Nevelsteen et al. 1995; Ryan et al. 2004).

Tissue-engineered grafts are ideal solution to above mentioned issue as they are biocompatible and promote cell adhesion and proliferation. Living cells are seeded on three-dimensional scaffolds, and then the scaffold can be transplant in the body via surgery. Huang et al. (2011) prepared scaffolds for vascular grafts by electrospinning collagen-chitosan-thermoplastic urethane blend. Glutaraldehyde was used for cross-linking the scaffolds to keep them from dissolving in culture medium. This scaffold was reported to have flexibility, high tensile strength, and biocompatibility. Tillman et al. (2009) synthesized polycaprolactone/collagen scaffolds which supported growth of vascular cells and retained their structural integrity over a month when implanted in vivo.

Nerve Regeneration

Autograft is the principle method for nerve regeneration. However it has several limitations such as donor site morbidity and lack of supply (Mackinnon and Hudson 1992). Several alternative options are being explored and nanofibrous scaffolds are one of them. Electrospun nanofibrous scaffolds has been evaluated for their efficacy in nerve regeneration due to their biocompatibility. Yang et al. (2005) developed aligned nanofibers by electrospinning of poly(L-lactic acid) and seeded the scaf-

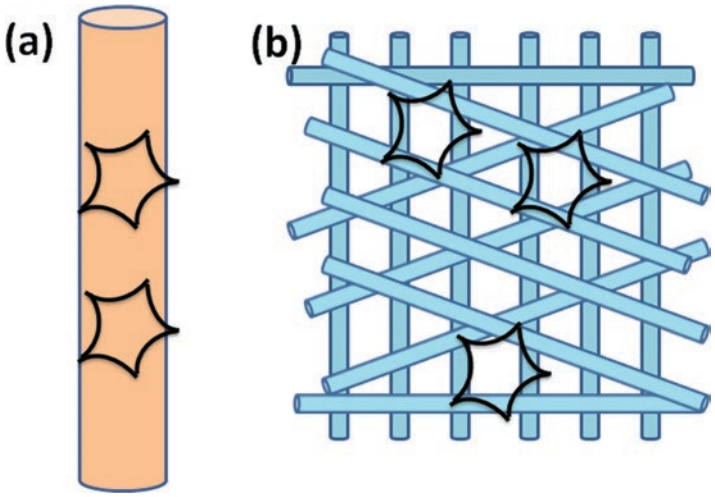

Fig. 7.4 Adherence, growth, and proliferation of cells on (**a**) microfibers and (**b**) nanofibers

folds with neural stem cells. They reported that the neurons grow well and cell elongation occurred along the nanofiber direction.

Significant research has been carried out in the field of nanofiber preparation and their utilization as scaffold material. There are still some challenges regarding adherence, growth, and proliferation of seeded cells on the nanofibers that need to be overcome. There are various factors that can influence the cellular compatibility like orientation of nanofibers, porosity, pore size, and nanophase surface roughness. Smaller size of nanofibers as compared to the cell size facilitates the organization of cells around nanofibers. The adherence, growth, infiltration, and spreading of cells on and between the nanofibers are higher than that on microfibers (Fig. 7.4) due to their high surface area and porosity. The quality and orientation of nanofibers also affect the cellular response. It is observed that nanophase roughness also promotes the cell adhesion. Cells adherence on the nanofibrous mats having smooth morphology is significantly higher than that of beaded morphology. Significant research has been carried out to study the role of nanofibers diameter on the adherence, growth, and proliferation. However the effect of fiber orientation and pore size on cell adhesion is still unexplored. Wang et al. (2018) investigated the cell adherence on aligned and randomly oriented electrospun dextran nanofibers. The cell morphology changed from spherical to elongated when the pore area of randomly oriented fiber membrane decreased, whereas in the case of aligned fiber membrane, the cell remained in elongated state on decreasing the pore area and was found to spread along the direction of alignment.

7.3.2 Wound Dressing

Wound dressing should have high water retention property, high water vapor transmission rate, nontoxic, and antibacterial properties. It should also show hemostatic ability, high absorption of exudates, and should be easy and painless to remove. The biopolymeric nanofibrous mats prepared with electrospinning are suitable for this purpose as they have small pore size and prevent bacterial invasion into the wounded area. With the advent of technology, it has become possible to electrospun biopolymers directly onto the wounded area which helps in normal skin growth and elimination of scar tissues (Jin et al. 2002; Smith and Reneker 2001). It has also been reported that electrospun nanofibrous mats of collagen type I show better wound healing capacity than the conventional counterpart (Smith et al. 2004; Wnek et al. 2003; Kenawy et al. 2003; Huang et al. 2003; Khil et al. 2003; Katti et al. 2004; Min et al. 2004a, b).

Other than collagen, various other biopolymers like chitosan and silk have also been used as wound dressing (Charernsriwilaiwat et al. (2012); Schneider et al. 2009). Ju et al. (2016) prepared wound dressing for burn wound healing by electrospinning silk fibroin. Unnithan et al. (2014) electrospun blend of cellulose acetate and polyurethane doped with zein powder and prepared wound dressing for burn, chronic, and diabetic wound infection. Chitosan-ethylenediaminetetraacetic acid/polyvinyl alcohol mats doped with *Garcinia mangostana* extracts have also been reported to possess antibacterial properties ideal for wound dressing (Charernsriwilaiwat et al. 2013).

7.3.3 Drug Delivery

Nanofibrous mats can be used as drug delivery vehicles for carrying drugs to targeted areas. They are preferred over conventional drug delivery system as they have high loading capacity, high encapsulation efficiency and are cost-effective (Wang et al. 2010; Chakraborty et al. 2009). These nanofibrous mats can be used for delivering drugs in a tuned manner. Degree of porosity, geometry, morphology of nanofiber and the type of polymer used affect the release of drug from the nanofiber (Goonoo et al. 2014). The dissolution rate of drug increases with increase in surface area and porosity of the carrier, i.e., nanofiber. This makes nanofibers in designing of a drug delivery system. Electrospinning offers a wide range of materials and drugs for the synthesis of drug delivery system. Electrospun nanofibrous mats have been used to deliver a variety of drugs as well as proteins, DNA, and RNA (Zamani et al. 2013). Both natural and synthetic polymers have been used as carrier in drug delivery system. Natural polymers are preferred due to their biocompatibility and low immunogenicity. Drugs can be incorporated into the polymer carrier either

directly or indirectly. Direct method is convenient and widely used. In this method, the drug is incorporated by homogeneously by dispersing it in the polymer solution followed by electrospun. Drugs which can't be homogeneously dispersed in the polymer solution require surface treatment. Surface treatment allows formation of nanoparticles on the nanofiber surface or within it which depends on the method used. Surface treatment is generally carried out by dipping the polymer nanofibers in the colloidal dispersion of drug nanoparticles which leads to adsorption on the surface of nanofibrous mats. Meng et al. (2010) synthesized fenbufen-loaded poly(D,L-lactide-co-glycolide)/gelatin nanofibrous scaffolds via electrospinning. They reported that the release rate of drug increases with increase in gelatin content. The release rate of drug was higher for randomly oriented scaffolds and was affected by change in pH. Elakkiya et al. (2014) prepared curcumin-incorporated silk nanofibers via electrospinning with average fiber diameter in range of 50–200 nm for drug delivery applications.

Some nanofibers along with their electrospinning conditions and applications have been summarized in Table 7.1.

7.4 Applications of Co-spun Nanofibers

The materials obtained by electrospinning have properties that are hard to obtain by conventional spinning. Co-electrospinning technique has been utilized for encapsulation of drugs in fiber core (Mickova et al. 2012), control release of drug (Zhang et al. 2004) and protects drug from harsh solvent environment of the polymeric solution in shell. It can also be used to produce cell-bearing composite micro threads in which the cell suspension inside the core is protected by shell from cellular damage during the fabrication process (Townsend-Nicholson and Jayasinghe 2006).

The core-sheath nanofibers from biodegradable polymers having polycaprolactone as sheath and gelatin as core have been prepared successfully by Zhang et al. (2004). Sun et al. (2006) reported the applications of material composed of poly(vinylpyrrolidone) (core) and poly(D,L-lactide) (sheath) for potential drug delivery. Collagen sheath with polycaprolactone core was found to have structure which favors fibroblast cell proliferation and its migration into the scaffold (Zhang et al. 2005b). The co-electrospinning has also been used for the formation of hollow nanotubes, prepared by the chemical removal of core (Li and Xia 2004a, b; Li et al. 2005a, b; Kalra et al. 2006; Gu et al. 2007; Zhan et al. 2007; Gu and Jian 2008; Di et al. 2008). Co-electrospinning modifies wettability properties of nanofiber surfaces and can be used to develop cell scaffolds, drug-releasing implants, and water-soluble bioactive agents.

Some core shell nanofibers along with their diameters and applications have been summarized in Table 7.2.

Table 7.1 Different processing parameters of biopolymer-based electrospun nanofibers and their potential applications in the field of regenerative medicine

S. No.	Biopolymer	Solvent	Applied voltage	Tip to collector distance	Diameter of nanofibers	Applications	References
1.	Chitosan-ethylenediaminetetraacetic acid/polyvinyl alcohol (with *Garcinia mangostana* extracts)	Distilled water	15 kV	20 cm	20,000–30,000 nm	Wound dressing	Charernsriwilaiwat et al. (2013)
2.	Chitosan-ethylenediaminetetraacetic acid/polyvinyl alcohol with lysozyme	Distilled water	15 kV	20 cm	143–209 nm	Wound dressing	Charernsriwilaiwat et al. (2012)
3.	Polyurethane/gelatin	1,1,1,3,3,3-Hexafluoroisopropanol	18 kV	15 cm	400–2100 nm	Wound dressing	Kim et al. (2009)
4.	Polycaprolactone/gelatin	Trifluoroethanol	10.5 kV	15 cm	500 nm	Wound dressing	Chong et al. (2007)
5.	Poly(D,L-lactide-co-glycolide)/collagen	1,1,1,3,3,3-Hexafluoroisopropanol	15–20 kV	15 cm	250 nm	Wound dressing	Liu et al. (2010)
6.	Chitosan/polyvinyl alcohol	Hydroxybenzotriazole, thiamine pyrophosphate, ethylenediaminetetraacetic acid in distilled water	15 kV	20 cm	20–30 μm	Wound dressing	Charernsriwilaiwat et al. (2014)
7.	Silk fibroin/polyethylene oxide	Epidermal growth factor, distilled Water	10–11 kV	15–21 cm	300 μm	Wound dressing	Schneider et al. (2009)
8.	Silk fibroin	Formic acid	15 kV	7 cm	80 nm	Wound dressing	Min et al. (2004b)
9.	Silk fibroin	Calcium chloride solution in ethanol and distilled water	2 kV	15 cm	180 μm	Burn wound dressing	Ju et al. (2016)
10.	Cellulose acetate/polyurethane/zein powder	N,N-Dimethylformamide/2-butanone	18 kV	15 cm	400–700 nm	Wound dressing for burn, chronic, and diabetic wound infection	Unnithan et al. (2014)

11.	Chitosan/silver nanoparticles	Trifluoroacetic acid/dichloromethane	23 kV	15 cm	126 ± 28 nm	Wound dressing	Lee et al. (2014)
12.	Chitosan/polyethylene oxide/poly(hexamethylene biguanide) hydrochloride	Acetic acid	8 kV	13 cm	60 nm	Wound dressing	Dilamian et al. (2013)
13.	Gelatin/silver nanoparticles	Acetic acid	15 kV	20 cm	280 nm	Wound dressing for burn wounds	Rujitanaroj et al. (2008)
14.	Silk fibroin/polyethyleneimine	Formic acid	17 kV	13 cm	236 ± 9 nm	Wound dressing	Çalamak et al. (2014)
15.	Collagen-polyethylene oxide	1,1,1,3,3,3-Hexafluoroisopropanol	15–20 kV	8 cm	460 nm	Wound dressing Tissue engineering	Rho et al. (2006)
16.	Chitosan-polyvinyl alcohol	Acetic acid solution	22 kV	10 cm	240 nm	Skin regeneration Scaffolds for tissue engineering	Kang et al. (2010)
17.	Carboxyethyl chitosan/ polyvinyl alcohol	Distilled water	25 kV	12 cm	131–456 nm	Skin regeneration	Zhou et al. (2008)
18.	Collagen/chitosan/ polyethylene oxide	Acetic acid	20–34 kV	10–25 cm	450 nm	Skin regeneration	Chen et al. (2008)
19.	Silk fibroin/curcumin	Trifluoroacetic acid	26 kV	14 cm	50–200 nm	Drug delivery	Elakkiya et al. (2014)
20.	Poly(D,L-lactide-co-glycolide)/gelatin	Trifluoroethanol	10 kV	10 cm	313 ± 69 nm	Drug delivery	Meng et al. (2011)
21.	Silk fibroin/graphene	Formic acid	19 kV	13 cm	400–800 nm	Bone regeneration	Nalvuran et al. (2018)
22.	Silk fibroin/$CoFe_2O_4$ or Fe_3O_4 nanoparticles	Formic acid	15 kV	15 cm	294 ± 53 nm	Bone regeneration	Brito-Pereira et al. (2018)

(continued)

Table 7.1 (continued)

S. No.	Biopolymer	Solvent	Applied voltage	Tip to collector distance	Diameter of nanofibers	Applications	References
23.	Hydroxyapatite/chitosan/ polyethylene oxide	Acetic acid/dimethyl sulfoxide	17.5 kV	34 cm	214 ± 25 nm	Bone regeneration	Zhang et al. (2008)
24.	Polyvinyl alcohol/collagen/ hydroxyapatite nanoparticles	Deionized water	3–20 kV	15 cm	320 nm	Bone regeneration	Asran et al. (2010)
25.	Gelatin	Acetic acid/ethyl acetate/ distilled water	12 kV	8 cm	40–670 nm	Bone regeneration matrix	Song et al. (2008)
26.	Gelatin/apatite/poly(L-lactic acid)-co-poly-(ϵ-caprolactone)	Trifluoroethanol	10 kV	15 cm	291 ± 51 nm	Bone regeneration matrix	Jegal et al. (2011)
27.	Poly(D,L-lactide-co-glycolide)/gelatin	Trifluoroethanol	7 kV	10 cm	568 ± 280 nm	Bone regeneration	Meng et al. (2010)
28.	Poly(D,L-lactide-co-glycolide)/collagen	1,1,1,3,3,3-Hexafluoro-2-propanol	8–10 kV	15 cm	269 nm	Bone regeneration	Jose et al. (2009)
29.	Polycaprolactone	Chloroform	13 kV	–	400 ± 200 nm	Bone regeneration	Yoshimoto et al. (2003)
30.	Poly(L-lactic acid)/collagen/ hydroxyapatite	1,1,1,3,3,3-Hexafluoro-2-propanol	12 kV	15 cm	310 ± 125 nm	Bone regeneration	Prabhakaran et al. (2009)
31.	Polycaprolactone/gelatin/ metronidazole	Trifluoroethanol/acetic acid	8–12 kV	20 cm	0.97 ± 0.20 μm	Anti-infective tissue regeneration membranes	Xue et al. (2014)
32.	Gelatin	Distilled water	22 kV	12 cm	211 nm	Periodontal tissue regeneration	Zhang et al. (2009)
33.	Gelatin/glyceraldehyde (cross-linker)	Distilled water/formic acid	25 kV	10 cm	95.19 nm	Cartilage tissue engineering	Aliakbarshirazi and Talebian (2017)
34.	Gelatin/polycaprolactone	Acetic acid/trifluoroethanol	10 kV	12 cm	440 ± 63 nm	Cartilage tissue engineering	Xue et al. (2013)

35.	Silk fibroin/L-lysine diisocyanate poly(ester-urethane)urea elastomer	1,1,1,3,3,3-Hexafluoroisopropanol	10 kV	15 cm	224 nm	Heart valve tissue engineering	Du et al. (2018)
36.	Polycaprolactone/collagen	1,1,1,3,3,3-Hexafluoro-2-propanol	20 kV	10 cm	_	Scaffolds in vascular reconstruction	Tillman et al. (2009)
37.	Collagen/thermoplastic urethane/chitosan/ glutaraldehyde (cross-linker)	1,1,1,3,3,3-Hexafluoro-2-propanol/trifluoroacetic acid	18 kV	12–15 cm	360 ± 20 nm	Scaffolds for vascular grafts and nerve conduits	Huang et al. (2011)
38.	Poly(L-lactic acid)	Dichloromethane/N,N-dimethylformamide	12 kV	10 cm	700 nm	Neural tissue engineering	Yang et al. (2005)
39.	Collagen/chitosan/ glutaraldehyde (cross-linker	1,1,1,3,3,3-Hexafluoroisopropanol	16 kV	130 mm	515 ± 253 nm	Scaffolds for smooth muscle cell	Chen et al. (2010b)
40.	Polycaprolactone/collagen	1,1,1,3,3,3-Hexafluoro-2-propanol	20 kV	10 cm	334 ± 125 nm	Scaffolds for muscle tissue	Choi et al. (2008)
41.	Hydroxyapatite/chitosan/ genipin	Trifluoroacetic acid	15 kV	15 cm	335 ± 119 nm	Repair and regeneration of maxillofacial defects and injuries	Frohbergh et al. (2012)
42.	Tropoelastin/collagen/ glutaraldehyde (cross-linker)	1,1,1,3,3,3-Hexafluoro-2-propanol	20 kV	20 cm	6.5 ± 1.7 μm	Scaffolds for dermal tissue engineering	Rnjak-Kovacina et al. (2012)
42.	Silk fibroin/polyethylene oxide	Calcium chloride/water/ethanol	20 kV	15 cm	_	Skin tissue engineering	Park et al. (2016)

(continued)

Table 7.1 (continued)

S. No.	Biopolymer	Solvent	Applied voltage	Tip to collector distance	Diameter of nanofibers	Applications	References
43.	Carboxymethyl chitin/ polyvinyl alcohol/ glutaraldehyde (cross-linker)	Distilled water	10 kV	8 cm	_	Stem cell tissue engineering	Shalumon et al. (2009)
44.	Cellulose acetate/carbon nanotubes	Acetone/N, N-dimethylformamide	20 kV	20 cm	305 ± 128 nm	Scaffolds for tissue engineering	Luo et al. (2013)
45.	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate/collagen	1,1,1,3,3,3-Hexafluoroisopropanol	12 kV	22 cm	300–600 nm	Scaffolds for tissue engineering	Meng et al. (2007)
46.	Chitin/silk fibroin	1,1,1,3,3,3-Hexafluoroisopropanol	17 kV	7 cm	340–920 nm	Scaffolds for tissue engineering	Park et al. (2006)

Table 7.2 Different applications of biopolymer-based core shell nanofibers

S. No	Core	Shell	Diameter	Application	References
1.	Polylactic acid	Chitosan	303 nm	Antibacterial material	Nguyen et al. (2011)
2.	Polyethylene oxide	Chitosan	150–190 nm	Wound dressing	Pakravan et al. (2012)
3.	Polycaprolactone	Silk fibroin	274 ± 92 nm	Skin tissue engineering	Li et al. (2011)
4.	Polycaprolactone	Collagen	385 ± 82 nm	Skin tissue engineering	Zhang et al. (2005b)
5.	Gelatin	Polycaprolactone	100–300 nm	Drug delivery	Zhang et al. (2004)
6.	Polyvinyl alcohol with embedded liposomes	Polycaprolactone	100 nm	Drug delivery	Mickova et al. (2012)
7.	Poly(L-lactic acid)	Chitosan	–	Drug carrier	Ji et al. (2013)
8.	Poly(D,L-lactide-co-glycolide)	Alginate	–	Growth factor delivery system	Choi et al. (2010)
9.	Poly(glycerol sebacate)	Gelatin	1 ± 0.125 μm	Regeneration of myocardial infraction	Ravichandran et al. (2011)
10.	Laminin	Poly(L-lactic acid)-co-poly-(ϵ-caprolactone)	316 ± 110 nm	Nerve tissue engineering	Kijeńska et al. (2014)
11.	Bone morphogenetic protein 2	Poly(L-lactic acid)-co-poly-(ϵ-caprolactone)-collagen/ dexamethasone	336.8 nm	Bone tissue engineering	Su et al. (2012)
12.	Polyvinyl alcohol with recombinant fibronectin/ cadherin 11	Poly(D,L-lactide-co-glycolide)/ collagen	465 ± 138 nm	Bone tissue engineering	Wang et al. (2014)
13.	Hydroxyapatite	Tussah silk fibroin	570–840 nm	Bone tissue engineering	Shao et al. (2016)
14.	Thermoplastic urethane	Collagen	0.72 μm	Scaffolds for tissue engineering	Chen et al. (2010a)
15.	Poly(glycerol sebacate)/ poly(L-lactic acid)	Poly(L-lactic acid)	450 ± 180 nm	Scaffolds for tissue engineering	Yi and LaVan (2008)

(continued)

Table 7.2 (continued)

S. No	Core	Shell	Diameter	Application	References
16.	Polycaprolactone	Gelatin	3.08 μm	Scaffolds for tissue engineering	Zhao et al. (2007)
17.	Sodium alginate	Polyethylene oxide	100–300 nm	Scaffolds for skin tissue engineering	Ma et al. (2012)
18.	Poly(glycerol sebacate)	Fibrinogen	1076 ± 212 nm	Cardiac regeneration	Ravichandran et al. (2013)
19.	Bovine serum albumin/ concentrated epidermal induction medium	Gelatin/poly(L-lactic acid)-co-poly-(ε-caprolactone)	366 ± 125 nm	Wound healing and skin reconstruction	Jin et al. (2013)

7.5 Applications of Melt Spun Nanofibers

Polymers which are insoluble in solvent at room temperature such as polyethylene, polypropylene, and polyether ether ketone cannot be processed by conventional solution electrospinning. For such polymers, melt electrospinning would be a good option. The flight path of melt jet does not have bending instabilities; therefore, it is reasonably stable in melt electrospinning than the solution electrospinning leading to the possibility of fiber deposition and organizing, which allows improved control over fiber placement. The technique deposits polymer fibers on the collector, while the collector is moving with certain speed, and melt electrospun fibers can be collected in layers upon layers also known as 3D printing. Melt electrospinning has many advantages over solution electrospinning. It has been reported that solution electrospinning is not appropriate for in vitro use directly onto cells because residual solvents can be toxic to tissues or cells (Dalton et al. 2006), and thus these solvents have to be fully removed (Min et al. 2004a). Hence, melt electrospinning can be used for preparations of fibers which have applications in tissue engineering and drug delivery. As melt electrospinning does not require removal of solvent, it has lower production costs and much higher production efficiency (Huang et al. 2012).

Despite being advantageous over solution electrospinning, the area of melt electrospinning is little explored. This is probably due to complex instrumentation (Ogata et al. 2007a), electric discharge problem due to the equipment design (Ogata et al. 2007b), high viscosity and low electrical conductance of polymer (Zhou et al. 2006). Melt spinning method is not appropriate for biopolymers such as collagen since it degrade at high temperature (Ogata et al. 2007b; Tian et al. 2009). Few synthetic biopolymers like poly(D,L-lactide-co-glycolide) and poly(L-lactic acid) have also been processed by electrospinning machine. However there is still a need to commercialize these biopolymer-based regenerative medicines.

7.6 Existing Tissue-Engineered Constructs and Regenerative Medicines in the Market

With the enormous growth in the field of tissue engineering and regenerative medicine, researches have been translated in the form of different products. There are various tissue-engineered construct or regenerative medicines available in the market, some of which are listed in Table 7.3.

Table 7.3 Various tissue-engineered constructs or regenerative medicines available in the market

S. No	Company name	Product name	Chemical composition	Applications
1.	DSM	PEA	Polyesteramide	Drug delivery
2.	DSM	Medeor® Matrix	Porcine dermis	Extracellular matrix
3.	DSM	Meso BioMatrix® Surgical Mesh	Porcine mesothelium	Extracellular matrix
4.	DSM	OsseoFit™	Collagen based	Implant
5.	Molnlycke	Mepilex®	Foam based	Wound dressing
6.	Molnlycke	Mepitel®	Polyamide net coated with soft silicone	Wound dressing
7.	Molnlycke	Mepiform®	Viscose based	Wound dressing
8.	Molnlycke	Mepitac®	Nonwoven fabric coated on one surface with a semipermeable polyurethane membrane	Wound dressing
9.	Aspen Medical Europe Ltd	Mesitran	Hydrogel sheet with honey	Wound dressing
10.	Hollister	CalciCare Calcium Alginate Dressing	Calcium alginate based	Wound dressing
11.	Hollister	CalciCare Calcium Alginate – Silver	Calcium alginate based	Wound dressing
12.	Hollister	Restore Foam Dressing with Silicone	Foam based	Wound dressing
13.	3 M	Tegaderm™ Silicone Foam Dressings	Foam based	Wound dressing
14.	Coloplast	Biatain® Silicone	Foam based	Wound dressing
15.	Coloplast	Biatain® Silicone Ag	Foam based	Wound dressing
16.	Coloplast	Comfeel®	Semipermeable polyurethane film coated with a flexible, cross-linked adhesive mass containing sodium carboxymethylcellulose and calcium alginate	Hydrocolloid dressing
17.	Organogenesis	PuraPly® Antimicrobial	Collagen sheet coated with 0.1% polyhexmethylenebiguanide hydrochloride	Wound dressing
18.	Organogenesis	PuraPly®	Native collagen matrix	Wound dressing

(continued)

Table 7.3 (continued)

S. No	Company name	Product name	Chemical composition	Applications
19.	Organogenesis	Affinity®	Amniotic membrane	Wound dressing
20.	Organogenesis	NuShield®	Placental allograft	Wound dressing
21.	Organogenesis	Apligraf®	AllohF in collagen gel plus stratified allohK	Temporary skin substitutes
22.	Organogenesis	Dermagraft®	AllohF on poly-galactin mesh	Temporary skin substitutes
23.	StrataTech	StrataGraft®	AllohF in collagen gel plus stratified allohK	Temporary skin substitute
24.	BSN medical	Elastomull® Elastic Gauze Bandage	42% cotton, 29% viscose, and 29% polyamide	Wound dressing
25.	BSN medical	Cover-Roll® Stretch Non-Woven Adhesive Bandages	Polyester based	Wound dressing
26.	BSN medical	Cuticell®	Acetate fiber based	Wound dressing
27.	BSN medical	Leukomed® Absorbent Wound Dressing	Viscose based	Wound dressing
28.	BSN medical	Leukomed® T Transparent Wound Dressing	Polyurethane based	Wound dressing
29.	BSN medical	Leukomed® T Plus Absorbent Film Wound Dressing	Viscose based	Wound dressing
30.	Avita Medical	ReCell®	Uncultured suspension of auto hK, delivered as a spray	Autograft
31.	Vericel Corporation	EpiCel®	Cultured auto hK multilayer sheet	Autograft
32.	RenovaCare	CellMist™	Liquid suspension containing a patient's own regenerative skin stem cells	Autograft
33.	Anika Therapeutics Inc.	Hyalomatrix®	Hyaluronic acid ester matrix	Wound care device
34.	Biohorizons	AlloDerm™	Decellularized human dermis	Acellular dermal matrix
35.	Integra LifeSciences Corp.	Integra® Dermal Regeneration Template	Bovine collagen and chondroitin sulfate coated with silicone	Skin regeneration template

(continued)

Table 7.3 (continued)

S. No	Company name	Product name	Chemical composition	Applications
36.	Integra LifeSciences Corp.	Integra® Meshed Dermal Regeneration Template	Collagen based	Skin regeneration template
37.	Integra LifeSciences Corp.	Integra® Bilayer Wound Matrix	Collagen, glycosaminoglycan, and polysiloxane	Wound care device
38.	Integra LifeSciences Corp.	Integra® Meshed Bilayer Wound Matrix	Collagen, glycosaminoglycan, and polysiloxane	Wound care device
39.	Integra LifeSciences Corp.	Integra® Wound Matrix	Collagen and glycosaminoglycan	Wound care device
40.	Integra LifeSciences Corp.	Integra® Flowable Wound Matrix	Collagen and glycosaminoglycan	Wound care device
41.	Integra LifeSciences Corp.	Integra® Wound Matrix (Thin)	Collagen and glycosaminoglycan	Wound care device
42.	Integra LifeSciences Corp.	Algicell®	Alginate based	Wound dressing
43.	Integra LifeSciences Corp.	Amniomatrix®	Cryopreserved liquid allograft derived from the components of the amniotic membrane and amniotic fluid	Allograft
44.	Integra LifeSciences Corp.	Alloskin™	–	Allograft
45.	Integra LifeSciences Corp.	AquaSite®	Hydrogel based	Wound dressing
46.	3 M Health Care	Tegaderm	Hydrogel based	Wound dressing
47.	ConvaTec	Aquacell	Sodium carboxymethylcellulose	Wound dressing
48.	Smith & Nephew Healthcare Limited	Acticoat	Silver-coated polyethylene mesh based	Wound dressing
49.	Smith & Nephew Healthcare Limited	Allevyn	Polyurethane foam based	Wound dressing
50.	Symatese	Nevelia	Collagen based	Dermal substitute

7.7 Conclusion Remarks and Future Prospects

Electrospinning is a versatile, easy, and cost-effective method of generating nonwoven fibers with high surface-to-volume ratio and porosity. Electrospinning can generate nanofibers on large scale by optimizing various important parameters such as concentration of dope solution, tip to collector distance, spinning rate, and voltage. Core-sheath nanofibers can be prepared using coaxial spinning, while aligned nanofibers can easily be prepared using high-speed rotating drum or magnetic field. Electrospinning nanofibrous mats have been successfully prepared using various natural biopolymers (collagen, gelatin, chitosan, and silk fibroin) as well as synthetic biopolymers (polylactic acid, polycaprolactone, and poly(D,L-lactide-co-glycolide)) which have also been approved by the Federal Drug Administration as biocompatible and biodegradable matrices for tissue engineering and regenerative medicine. These nanofibrous mats have very large surface area, porosity, and interconnected morphology that allow passage of gases, nutrients, and metabolites which favor seeded cells to grow and proliferate faster. The biocompatibility of nanofibrous mats was further enhanced by functionalization with different growth factors and enzymes which can regulate the adherence, growth, proliferation, and differentiation of seeded cells. These impressive innovations in nanofibrous matrices make it suitable candidates for tissue engineering and regenerative medicine. However there are several challanges. Low infiltration of cells seeded onto nanofibrous mats is the major problem which must be resolved before commercialization of the nanofiber matrices for tissue engineering and regenerative medicine. Some attempt has been made to mitigate this problem by enlarging the pore size of nanofibrous mats as well as successful spinning of live cell in core by coaxial electrospinning. The second problem is related to electrospinning of highly branched as well as charged polymers like pectin and chitosan which are very difficult to electrospun due to charge repulsion. Many researchers spun these materials by blending with other polymer having good electrospinnability. With theoretical modeling, we can easily optimize the various electrospinning process parameters and architecture of different biopolymers formulations. We can design the tissue-engineered construct that would be a promising material for tissue engineering and regenerative medicine by controlling morphological features and architecture of nanofibrous mats of various biocompatible polymers. In addition, coculturing the different cells in these engineered nanofibrous mats will help the complete construction of tissue. Although significant research has been carried out in the area of nanofibers manufacturing. However only few products have been commercialized. There is a huge future scope for optimization and successful commercialization of these materials for the benefits of human beings.

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Chapter 8
Solid Lipid Nanoparticles: A Multidimensional Drug Delivery System

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Contents

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Abstract The development of drug delivery carriers is often very challenging, due to the physicochemical properties of the drug such as poor solubility, low permeability, and short half-life. Development of colloidal delivery systems has introduced new avenues for safe and effective drug delivery. However, the absence of large-scale production methods, toxic solvent residuals, limited stability, and cytotoxicity of some polymeric particles are the major issues associated with colloidal carriers. On this note, the concept of lipid-based nanoparticles like solid lipid nanoparticles matured. Solid lipid nanoparticles not only combine the advantages of the conventional drug delivery systems but also bypass their major disadvantages.

Solid lipid nanoparticles represent an alternative carrier system to conventional colloidal carriers due to their specific features such as use of natural fabrication components, size and related narrow distribution, enhanced stability, and increased permeation through biological barriers. Additionally, increased solubility, biocompatibility, ease of manufacture, and different possible administration routes enable solid lipid nanoparticles a frontline drug delivery system. Here, I reviewed up-to-date developments about solid lipid nanoparticles as a potential nanocolloidal system for drug delivery. The major points are as follows: (1) overview of the different production methods, which are suitable for large-scale production, and analytical techniques used for characterization of solid lipid nanoparticles are described; (2) in vitro evaluation, pharmacokinetics, and tissue distribution of solid lipid nanoparticles; and (3) stability, toxicity, and status of excipients used in the fabrication of solid lipid nanoparticles have been discussed in this chapter. This chapter selectively highlights major therapeutic applications of solid lipid nanoparticles in drug delivery along with mechanism of action of the incorporated drug molecule.

Keywords Solid lipid nanoparticles · Pharmacokinetics · Novel drug delivery system · Colloidal drug carrier · Bioavailability · Biological barrier · Anticancer · Antitumor · Vaccine adjuvants

8.1 Introduction

Over the last 20 years, nanoscience has emerged as a novel interdisciplinary area of science, which has initiated the opportunity of research on the development of nanomaterial-based nanomedicine. Nanomedicine signifies potential biomedical and pharmaceutical applications as a novel drug delivery system, biosensing, and bioimaging. Nanomedicine affords controlled drug release and targeted delivery of active pharmaceutical ingredients at the site of action. Distinct varieties of colloidal drug delivery systems have been developed from nanomaterials such as liposomes, dendrimers, and polymeric nanoparticles. Polymeric nanoparticles, usually made with suitable biodegradable polymers such as the group of polyalkylcyanoacrylate and polymethyl methacrylate, have been proved to extend the release of the incor-

porated drugs (Blasi et al. 2007). Polymer system offers the advantage of chemical modifications, including the synthesis of block and copolymers. But, the lack of scale-up production methods, high cost of the polymeric components, toxic solvent residuals, cytotoxicity, and chemical obstacle of some polymeric particles (e.g., catalyst residues, molecular nonhomogeneity) are the significant problems associated with polymeric nanoparticles (Date et al. 2006).

During the last couple of decades, researchers have focused on the development of alternative carrier system to liposomes, emulsions, and polymeric nanoparticles, the so-called solid lipid nanoparticles, to overcome the abovementioned issues. Solid lipid nanoparticles represent a class of colloidal particles composed of lipids being solid in both room and body temperatures (Kumar et al. 2014). The scientists have utilized the fact that the use of solid lipids alternately to liquid oils may provide controlled drug release, as the fluidity of the drug in a solid lipid matrix is substantially lower as compared to liquid oil. The average diameter of solid lipid nanoparticles is in the submicron range from 50 to 1000 nm. They are composed of physiologically tolerated lipids dispersed in an aqueous surfactant phase (Gastaldi et al. 2014; Weber et al. 2014). Figure 8.1 illustrates the general structure of solid lipid nanoparticle. Solid lipid nanoparticles facilitate encapsulation of hydrophilic and hydrophobic drugs and shield them from degradation in the body and contributing their sustained release. Additionally, solid lipid nanoparticles are good cargo for delivery of cosmetic agents, vaccine, and biologically active food components. Table 8.1 summarizes the various advantages of pharmaceutical and biological

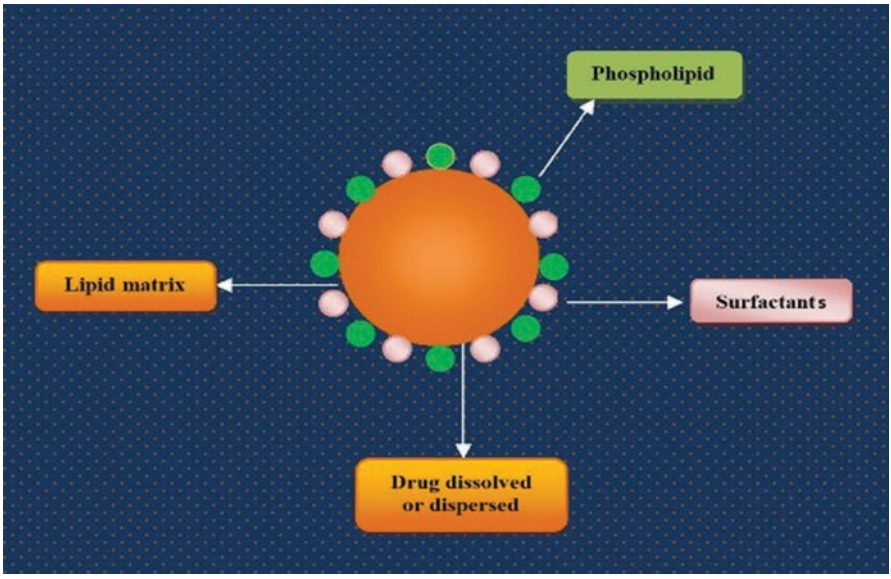

Fig. 8.1 General structure of solid lipid nanoparticle. Facilitate encapsulation of hydrophilic and hydrophobic drugs and contributing their sustained release by diffusion of drug molecules through lipid matrix and in vivo degradation of the lipid matrix

Table 8.1 Numerous advantageous features of solid lipid nanoparticles from pharmaceutical and biological aspects and few technological and biological drawbacks as the nanocolloidal drug delivery system

Characteristics	Pharmaceutical aspects	Examples	Perspectives	References
Advantages	Pharmaceutical	Physical stability	Increased drug stability in the formulation	Gastaldi et al. (2014)
		Incorporation of hydrophilic and hydrophobic drugs	Versatility of application as a carrier for drugs from different pharmacological groups	Soares et al. (2013)
		Production can be scaled up	Possibility of industrial production	Shegokar et al. (2011)
		Ease of manufacture	Possibility of fabrication in each laboratory, low cost	Yu et al. (2012)
		Avoidance of organic solvents	No toxicity concerns, green chemistry	Nafee et al. (2014)
		Ability to carry two active agents	Combined therapy	Yu et al. (2012)
		High drug entrapment efficiency	Minimization of formulation dose	Kheradmandnia et al. (2010)
		Possibility of sterilization	Suitable for parenteral administration	Mehnert and Mäder (2001)
	Biological	Administration via different routes	Increased spectrum of drug application, possibility of better selection of suitable therapy	Shegokar et al. (2011)
		Biodegradability	Possibility of application as a matrix for sustained drug release	Yu et al. (2012)
		Controlled drug release	Safety for patients, prolonged drug release	Xie et al. (2011)
		Site-specific targeting	Decrease of systemic toxicity, targeted therapy	Gastaldi et al. (2014)
		Biocompatibility	Avoidance of allergenic reactions	Silva et al. (2011)
		Increase of bioavailability of incorporated drug	Dose minimization	Dwivedi et al. (2014)
Disadvantages	Technological	Low drug-loading capacity	High doses of formulation are required	Almeida and Souto (2007)
	Biological	Initial burst effect of incorporated drug	Risk for patient resulting from overdosing	Kuo and Ko (2013)

aspects and few disadvantages of solid lipid nanoparticles. This chapter sets the scene for the discussion of the recent literature about solid lipid nanoparticles pharmaceutical and biomedical applications. Additionally, background information such as composition, production methods, characterization, and pharmacokinetics including in vitro evaluation of solid lipid nanoparticles is also described.

8.2 Formulation and Characterization of Solid Lipid Nanoparticles

Numerous formulations techniques have been practiced in solid lipid nanoparticles preparation. Among them, high-pressure homogenization and microemulsion techniques are generally preferred. Briefly, in high-pressure homogenization, the molten lipid blended with an aqueous surfactant solution followed by high-pressure homogenization. Subsequent cooling down of the mixture results in recrystallization of nanoemulsion and solid lipid nanoparticles formation. The microemulsion method composed of a dispersion of warm microemulsion of molten lipid and surfactant in cold water (Wissing et al. 2004). Solid lipid nanoparticles can be also prepared by using solvent emulsification method followed by solvent evaporation and diffusion. In the emulsification and solvent evaporation method, ordinarily water-immiscible organic solvents such as chloroform are used, whereas the emulsification and solvent diffusion technique involves the use of solvents like ethanol, acetone, or dimethyl sulfoxide. Consequent addition of water results in diffusion of organic solvent to aqueous phase and producing solid lipid nanoparticles precipitation (Venishetty et al. 2013). Furthermore, solid lipid nanoparticles can also be prepared using a double emulsion technique in which primary emulsion formed by homogenization and mixed with emulsifier forming w/o/w double emulsion (Zariwala et al. 2013). It should be noticed that the active compound is usually incorporated into the oil phase and occasionally to the aqueous phase (Xie et al. 2011). Emulsification technique offers the advantage of avoidance of elevated temperatures, while the high-pressure homogenization technique exhibited no issue with scaling up. An influential disadvantage of emulsification and solvent evaporation and double emulsion techniques is the usage of water-immiscible solvents such as chloroform, dichloromethane, or toluene, which are hazardous to human as well as to the environment. Particularly, chloroform and dichloromethane cause carcinogenicity; hence, their use should be avoided. The methods such as melt emulsification and hot melt homogenization require the use of high temperatures, which can degrade the incorporated thermolabile drugs or proteins.

The characterization of solid lipid nanoparticles dispersion provides precise information about prepared formulation (Shah et al. 2015). In-depth research of the nanocolloidal carriers has a crucial role in the formulation of solid lipid nanoparticles drug delivery systems. Certain characterization parameters help to predict the drug-loading capacity, drug release kinetics, stability study, and in vivo performance

Table 8.2 Most common analytical techniques used for evaluation of particle size and shape, polydispersity, zeta potential, and crystallinity of the solid lipid nanoparticles

Characterization technique	Parameter determined	References
Photon correlation spectroscopy (PCS)	Particle size, polydispersity, zeta potential	Venishetty et al. (2013)
Laser diffractometry (LD)	Particle size	Silva et al. (2011)
Differential scanning calorimetry (DSC)	Thermal properties/degree of crystallinity	Zariwala et al. (2013)
Scanning electron microscopy (SEM)	Surface morphology	Rostami et al. (2014)
Cryogenic field emission scanning electron microscopy	Surface morphology	Das et al. (2011)
Transmission electron microscopy (TEM)	Particle size and shape	Silva et al. (2011)
High-resolution transmission electron microscopy	Particle size	Dwivedi et al. (2014)
Scanning tunneling microscopy (STM)	Particle shape	Kelidari et al. (2015)
Atomic force microscopy (AFM)	Particle size and shape, textural properties	Akanda et al. (2015)
Cross-polarized light microscopy	Confirmation of drug encapsulation	Das et al. (2011)
X-ray diffraction (XRD)	Polymorphism and crystallinity confirmation	Venishetty et al. (2013)
Wide-angle X-ray scattering (WAXS)	Polymorphism and crystallinity confirmation	Silva et al. (2011)
X-ray photoelectron spectroscopy (XPS)	Confirmation of surface modification	Venishetty et al. (2012)
Fourier-transform infrared spectroscopy	Functional group analysis	Kelidari et al. (2015)

of solid lipid nanoparticles. Appropriate characterization of solid lipid nanoparticles dispersion addresses detailed information about prepared formulation. Table 8.2 reviewed the numerous common analytical techniques used for characterization of solid lipid nanoparticles. Various solid lipids such as mono-, di-, and triglycerides, fatty acids, waxes, and steroids are used for the formulation of solid lipid nanoparticles (Cai et al. 2011). For the stabilization purpose of solid lipid nanoparticles formulation, various surfactants are also incorporated. Furthermore, varying methods and parameters are required for homogenization of different lipids and emulsifying agent. Researchers should take consideration for proper selection of the emulsifier and lipids as they influence the stability of solid lipid nanoparticles. Muller et al. (2002) reported that a higher concentration of emulsifier reduces the particle size, which is beneficial for the purpose of intravenous administration. However, the incorporation of higher lipid content results in larger particle size. Table 8.3 summarizes the variety of components used for solid lipid nanoparticles fabrication. Recently, cationic solid lipid nanoparticles enriched with the advan-

Table 8.3 Various categories of excipients used in fabrication of solid lipid nanoparticles drug delivery system, solid lipid nanoparticles developed by solid lipid matrices, and emulsifier generally recognized as safe with respect to biocompatibility and nontoxicity (Mishra et al. 2018)

Excipients type	Examples
Lipid matrices	Beeswax, behenic acid, caprylic/capric triglyceride, glyceryl trimyristate, glyceryl monostearate, glyceryl tristearate, hardened fat (Witepsol E 85), solid paraffin, glyceryl behenate (Compritol), glyceryl tripalmitate
Emulsifiers	Phosphatidylcholine, poloxamer, poloxamine, polysorbate 80, egg lecithin
Co-emulsifiers	Tyloxapol, taurocholate sodium salt, sodium dodecyl sulfate, sodium glycocholate, sodium oleate, cholesteryl hemisuccinate, butanol
Cryoprotectants	Trehalose, glucose, mannose, maltose, lactose, sorbitol, mannitol, glycine, polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), gelatin, stearylamine
Charge modifiers	Stearylamine, dicetyl phosphate, dipalmitoylphosphatidylcholine (DPPC), dimyristoylphophatidylglycerol (DMPG)
Stealthing agents	Polyethylene glycol, poloxamer
Preservative	Thiomersal

tages of the lipid matrix and the hydrophilic layer have gathered increasing research attention. In comparison to traditional solid lipid nanoparticles, the surface of cationic solid lipid nanoparticles is positively charged as they contain cationic lipids or surfactants (Yu et al. 2012; Doktorovová et al. 2014). Cationic lipids are composed of the hydrophilic head (quaternary ammonium salt characterized by positively charged nitrogen atom) and the hydrophobic chain. In this occurrence, the positive charge results from protonation of amine groups at appropriately low pH. The lipid core of cationic solid lipid nanoparticles serves as a pool for hydrophobic drugs, while positively charged surface promotes better cellular internalization for tumor targeting, penetrating the blood-brain barrier, and improving gene transfection (Hwang et al. 2015).

8.3 Properties of Solid Lipid Nanoparticles

Solid lipid nanoparticles are enriched with diversified attributes of pharmaceutical and medical interest, which enable them as distinguished novel drug delivery system. The nanometric size range of solid lipid nanoparticles and use of Food and Drug Administration-approved lipids and surfactants generally recognized as safe status and their application via different routes of administration such as intravenous, oral, ocular, topical, inhalation, intranasal, rectal, subcutaneous, and intramuscular administration demonstrate their versatility (Mortiz-Geszke M and Mortiz M 2016). A suitable modification of solid lipid nanoparticle and their formulation by using physiological compounds enable them to cross different biological barriers and ensure the drug delivery at the targeted site as well as minimize the jeopardy of toxicity (Kakkar et al. 2011). Furthermore, a unique property of suitable surface

modification of solid lipid nanoparticles significantly lessens the primary prompt release of encapsulated drug, minimizing so-called burst effect. It has been mentioned that solid lipid nanoparticles composed of miscellaneous lipids exhibit higher drug-loading capacities as compared to those consisting of similar lipid molecules. The heterogeneity of lipid phase inhibits its crystallization during storage and hinders drug expulsion from solid lipid nanoparticles matrix (Shegokar et al. 2011; Ying et al. 2011). Solid lipid nanoparticles are usually stable for more than one year during storage. Usually, solid lipid nanoparticles formulation stored at 4 °C displays better stability as compared to formulations stored at room temperature. Therefore, Ravi et al. (2014) have prescribed storage of solid lipid nanoparticles under refrigerated conditions. Labovkina et al. (2011) reported that after administration to the body, solid lipid nanoparticles are removed from the circulation by the liver or spleen. Interestingly, rapid uptake of solid lipid nanoparticles can be avoided by their coating with poly(ethylene) glycol (PEG) leading to prolonged circulation time. Moreover, PEGylation has been reported to increase the diffusion ability of solid lipid nanoparticles across epithelium and to improve their stability in simulated body fluids. The drug release profile from solid lipid nanoparticles ordinarily reveals a biphasic pattern with initial burst effect followed by a prolonged release over several hours or days (Teskač and Kristl 2010). The primary release of the incorporated drug from solid lipid nanoparticles based on diffusion from the external particle surface or matrix erosion is induced by hydrolytic degradation. Consequently, active substance gradually released from the lipid core through diffusion and promotes extended release and dissolution. The rate of release may be influenced by the nature, composition of lipid matrix, and selection and concentration of surfactants including technological parameters (Kakkar et al. 2011).

8.4 In Vitro Evaluation of Solid Lipid Nanoparticles

8.4.1 Drug Release from Solid Lipid Nanoparticles

The drug entrapped in the solid lipid nanoparticles not always homogenously dissolved in the lipid matrix, and it could be localized in different regions of the particles (Fig. 8.2). Localization may alter drug release and biological characteristics of the product. In some instances, the drug release kinetics of the incorporated drug from the solid lipid nanoparticles can be modified by varying the production method or its parameters. Drug release from solid lipid nanoparticles is reliant on the diffusion of drug molecules through lipid matrix and in vivo degradation of the lipid matrix. In contrast to polymeric nanoparticles, lipid nanoparticles can be degraded by lipase in blood and provide drug release (Westesen and Siekmann 1996). This is evident from the burst release of tetracaine and etomidate solid lipid nanoparticles prepared from glyceryl monobehenate because the melting point of tetracaine and etomidate is lower than the melting point of lipids (Muller et al. 1994). In the pro-

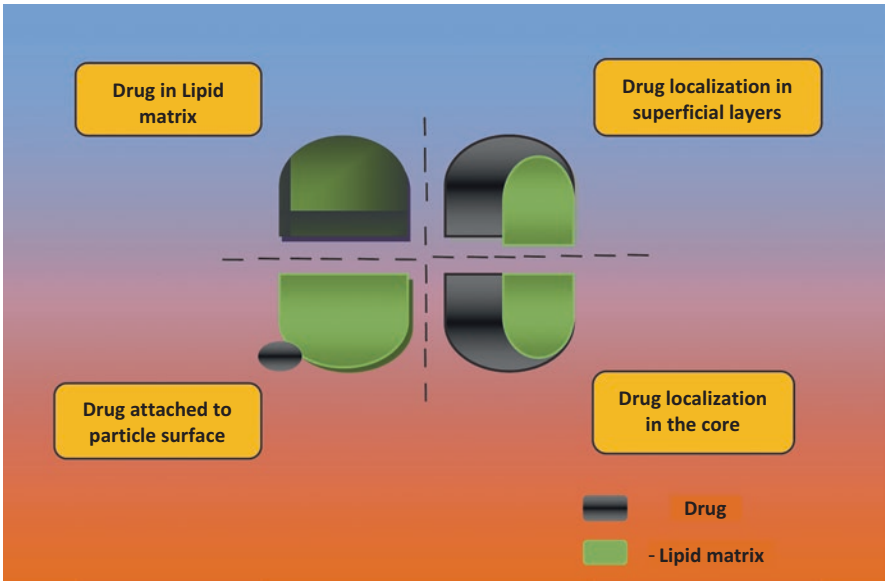

Fig. 8.2 Different drug localization patterns in lipid matrix of solid lipid nanoparticles. Localization affects drug release and biological properties of the formulation

duction of solid lipid nanoparticles during the cooling of homogenized mixture, lipids having high melting point solidify first and the drug localizes preferentially on the surface of solid lipid nanoparticles. In contrast to this, prednisolone-loaded solid lipid nanoparticles showed a distinctly prolonged release over a few weeks. After the homogenization process, prednisolone, a high melting point lipid (230 °C), precipitates first forming a drug core in the lipid phase. The diffusion velocity of the drug to the surface of solid lipid nanoparticles is very slow. Thus, sustained-release pattern was observed as a result of drug release study performed by using Franz diffusion cells separating the receptor fluid and the nanoparticle dispersion with a membrane (cellulose nitrate membrane of 0.1 milli pore diameter). Olbrich et al. (2002) studied the release of retinol from solid lipid nanoparticles, and they found that particles with low oil quantity in the lipid matrix composition showed significant controlled release. Similarly, Venkateswarlu and Manjunath (2004) have performed in vitro release studies on clozapine solid lipid nanoparticles of different triglycerides, using modified Franz diffusion cells. Researchers demonstrated that the amount of clozapine released from solid lipid nanoparticles was inversely related to the partition coefficient of clozapine in triglycerides used. The highest release percentage of clozapine was found in formulation whose particle size was least than the other. The solubility of the drug also affects the rate of release, probably through the partition coefficient. Additionally, the temperature applied during the formulation of the solid lipid nanoparticles has an impact on drug release. High temperatures solubilize the drug in the aqueous phase during manufacture and therefore

promote drug localization at the surface region. Few of the most frequently mentioned characteristics influencing the drug release appear to be particle size, effect of electrolyte concentration, and pH.

8.4.2 Effect of Electrolyte Concentration and pH

The advantages of solid lipid nanoparticles such as sustained release and improved bioavailability are associated with their size in the submicron range. The protection of the particle size of colloidal carrier systems after per oral administration is a critical point. It is a well-known fact that ionic strength and pH in the gastrointestinal tract affect the stability of solid lipid nanoparticles, when administered orally. High electrolyte concentrations dehydrate the adsorbed surfactant layer and thereby further diminish its thickness and stabilizing effect. Low pH has a stronger influence than that of ionic strength, in destabilizing the solid lipid nanoparticles dispersion. Freitas and Muller have studied the influence of different electrolytes on Compritol solid lipid nanoparticles. Results demonstrated that a pronounced destabilizing effect was observed with increasing electrolyte concentrations and increasing valency. Reduced electrostatic repulsions and gel formation are the key mechanisms involved in the destabilizing effect of the electrolyte (Freitas and Muller 1999a, b).

Zimmermann and Muller (2001) have examined the impact of the gastrointestinal medium on the in vitro physical stability of the solid lipid nanoparticles. Results showed that nonionic steric emulsifier, poloxamer 188, was suitable for stabilizing the triglyceride solid lipid nanoparticles in the artificial gastrointestinal medium at pH 1.1 but not for the lipid (Imwitor). Stabilizing properties certainly depend upon anchoring of the stabilizer on the lipid surface. However, stability studies in simulated conditions (containing degradation enzymes like lipase) need to be performed to foretell the stability of orally administered solid lipid nanoparticles.

8.4.3 Effect of Enzymes

Muller et al. (1995) investigated the effect of enzymes on in vitro degradation of solid lipid nanoparticles in solutions of pancreatic lipase/colipase and free fatty acids formed by using turbidimetry. Results showed that the degradation velocity has found to depend on the nature of the lipid matrix. The rate of the degradation is highest for trimyristin, medium for cetyl palmitate, and relatively slow for lipids with longer fatty acid chains, like tribehenin. The surfactants used for solid lipid nanoparticles stabilization had a dominating effect on degradation velocity. Poloxamer 188 could prevent the in vitro degradation of well-degradable trimyristin particles. This is due to the lack of adherence of the lipase to the particle surface, which is essential for enzymatic degradation. The dominating effect of the surfactant can be exploited to design solid lipid nanoparticles with optimum degradation

and matrix-controlled drug release. It has been proved that the longer the fatty acid chains in the glycerides, the slower the enzymatic degradation. Some surfactants can accelerate the degradation, and surfactant like poloxamer 407 sterically stabilizes solid lipid nanoparticles and retards the enzymatic degradation.

8.5 Pharmacokinetics and Tissue Distribution of Solid Lipid Nanoparticles

8.5.1 Intravenous Administration

Intravenous administration is the desired route in various fatal disorders to produce immediate therapeutic benefit. Solid lipid nanoparticles serve as carrier for intravenous administration of certain drugs such as diazepam, which cause pain and inflammation at the site of injection, and for drugs like etoposide, which produce toxicity with excipients used to solubilize it. Yang and coworkers developed camptothecin-loaded solid lipid nanoparticles by high-pressure homogenization and injected them intravenously into mice. The results showed that in ingested organs, the area under curve, the dose, and the mean residence time of camptothecin-loaded solid lipid nanoparticles were much higher than camptothecin solution, especially in the brain, in the heart, and in organs containing reticuloendothelial cells. Among the tested organs, area under curve ratio of camptothecin-loaded solid lipid nanoparticles to camptothecin solution was highest in the brain. These results indicate that solid lipid nanoparticles are promising drug targeting systems for lipophilic antitumor drugs and may allow the reduction in dose and a decrease in systemic toxicity (Yang et al. 1999a, b).

Cavalli et al. (2002a, b) has reported that intravenous administration of paclitaxel-loaded solid lipid nanoparticles led to higher and prolonged plasma levels of paclitaxel. Both paclitaxel non-pegylated and pegylated solid lipid nanoparticles exhibited a low uptake by liver and spleen macrophages and increased uptake in brain. Likewise, doxorubicin pegylated and non-pegylated solid lipid nanoparticles provided higher concentrations of doxorubicin in the brain of two animal species (rats and rabbits) tested, following intravenous administration. The concentration of doxorubicin in the brain increased as the concentration of the pegylating agent increased. Cardiotoxicity of solid lipid nanoparticles was lower when compared with that of doxorubicin solution (Fundaro et al. 2000). Camptothecin-loaded solid lipid nanoparticles coated with poloxamer 188 produced by high-pressure homogenization were administered orally to mice. Reverse-phase high-performance liquid chromatographic studies revealed that there were two peaks in the camptothecin-loaded solid lipid nanoparticles. The first peak was the result of free drug, and the second peak was indicative of gut uptake of camptothecin-loaded solid lipid nanoparticles after 3 hours. In tested organs, the area under curve of camptothecin-loaded solid lipid nanoparticles and mean residence time increased significantly as

compared with the camptothecin solution, and among all the tested organs, the increase of area under curve was highest in the brain (Yang et al. 1999a, b). These results suggest that solid lipid nanoparticles could be a promising sustained release and targeting delivery system for camptothecin or other lipophilic antitumor drugs, after oral administration.

8.5.2 Topical Application

Solid lipid nanoparticles offer a number of advantages for the topical route of administration. Due to small particle size, solid lipid nanoparticles ensures close contact to stratum corneum and thereby enhances penetration of encapsulated drug into the viable skin. Sustained release of the drug from solid lipid nanoparticles supplies the drug to the skin over a prolonged period and thereby reduces systemic absorption. Solid lipid nanoparticles showed occlusive properties because of film formation on the skin, which reduces transdermal water loss (Fig. 8.3). The increase of water content in the skin reduces the indications of atopic eczema and improves the appearance of healthy human skin. Occlusion also favors drug penetration into the skin (Fig. 8.3). Jenning et al. (2000a, b) successfully incorporated vitamin A-loaded solid lipid nanoparticles in conventional topical dosage forms like hydro-

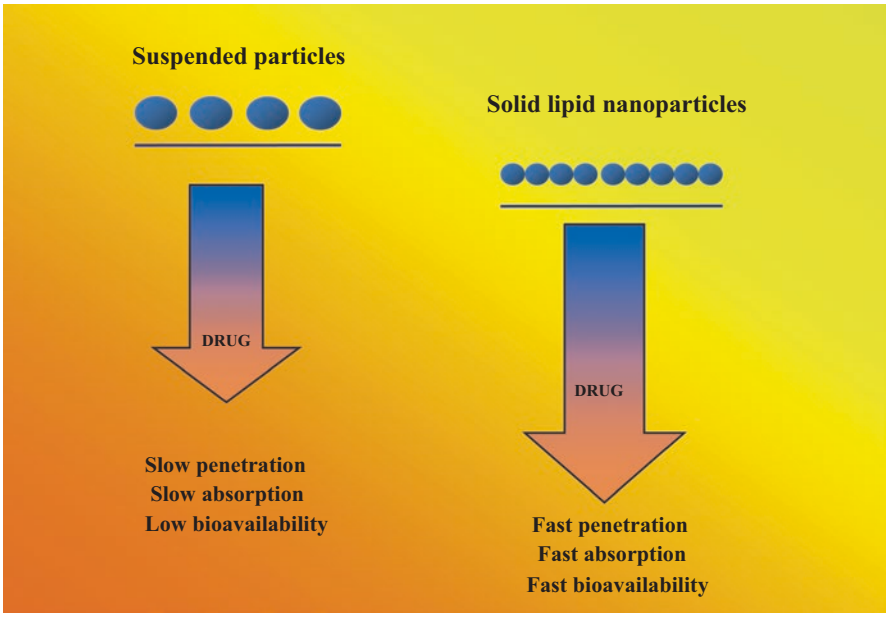

Fig. 8.3 Tighter distribution, film formation, and better penetration of solid lipid nanoparticles applied on skin compared to microparticles

gels and o/w creams. Drug release studies (using Franz diffusion cell) and differential scanning calorimetric studies showed good relationship between polymorphic transitions and prolonged drug release. Polymorphic transitions can induce by water evaporation from the suspensions and subsequent gel formation or by aggregation in buffer solution. Both factors must be considered in topical application. After applying the topical dosage form to the skin, water evaporates from the preparation and solid lipid nanoparticles are exposed to electrolytes present in the surface of the skin. These factors induce polymorphic transitions, and as a result, drug expulsion takes place.

Controlled release of drug from solid lipid nanoparticles can be achieved by controlling polymorphic transitions, using surfactant mixtures. Upon application, solid lipid nanoparticles transform slowly to the stable polymorph and sustain release is expected. Application of a conventional o/w cream did not change skin structure, whereas the application of solid lipid nanoparticles containing cream increased the thickness of the stratum corneum, which in turn improves the penetration barrier to the drug. Therefore, the penetration characteristics of the drug can be altered by incorporating them into solid lipid nanoparticles. In another study, the same researchers group applied solid lipid nanoparticles containing oil in water cream to porcine skin and studied the distribution of retinol in the skin. The outcomes of study were compared to those obtained from conventional nanoemulsions and hydrogels. Prolong targeting was achieved with solid lipid nanoparticles (retinol-loaded) incorporated into oil in water cream that slow down the polymorphic transition and, thus, drug expulsion. Thus, using solid lipid nanoparticles encapsulation, a drug localizing effect in the skin seems possible (Jenning et al. 2000a, b). Prednicarbate, a topical glucocorticoid, incorporated into solid lipid nanoparticles to get the drug targeted to viable epidermis. Results demonstrated that prednicarbate penetration into the human skin increased by 30% in the case of solid lipid nanoparticles when compared with cream (Maia et al. 2000). Chemically labile active ingredient (vitamin E) protected against degradation by incorporating them into solid lipid nanoparticles. The occlusion promotes the penetration of vitamin E into the skin, as shown by the stripping test (Dingler et al. 1999).

8.5.3 Ocular Administration

Solid lipid nanoparticles have been emerged as a promising drug delivery system for administration of ocular drugs such as pilocarpine and tobramycin. Solid lipid nanoparticles containing an ion-pair complex of tobramycin with hexadecyl phosphate (1,2 molar ratios) were developed by the warm o/w microemulsion method (Cavalli et al. 1995). Preocular retention of solid lipid nanoparticles was investigated by instilling fluorescent solid lipid nanoparticles dispersions into the lower conjunctional sac of rabbit eyes. The fluorescent solid lipid nanoparticles dispersions formed a stable precorneal film and were retained longer time in the eye. Tobramycin-loaded solid lipid nanoparticles were administered topically to the rab-

bits, and they produced significantly higher tobramycin bioavailability in the aqueous humor when compared with the standard commercial eye drops. The increased tobramycin availability in aqueous humor might be due to entrapment and prolonged retention of solid lipid nanoparticles in the mucin layer covering the corneal epithelium (Cavalli et al. 2002a, b).

8.6 Stability of Solid Lipid Nanoparticles

Stability is a prime concern as far as the pharmaceutical significance of dosages forms concerned either conventional or novel drug delivery system. Aqueous dispersions of solid lipid nanoparticles are stable up to 3 years, though in some systems, particle growth followed by gelation was noted on storage. Exposure to light, temperature, and degree of crystallinity are common factors, which influence the long-term stability of solid lipid nanoparticles. To examine the effect of light, solid lipid nanoparticles dispersion was filled into glass vials and stored at different light conditions (dark, daylight, and artificial illumination). Two fluorescent lamps were used to create artificial illumination. Storage in white glass vials under artificial light induced accelerated gelation. The gelation process was inattentive under daylight. It has been observed that an increase in the intensity of light radiation leads to accelerated particle growth and gelation. The brown glass absorbs the light at a short wavelength (300–600 nm) and prevents high energetic radiation from falling on solid lipid nanoparticles dispersions and, consequently, improved stability of solid lipid nanoparticles.

The effect of the temperature on solid lipid nanoparticles composed of Compritol lipid matrix was investigated by storing them at 88 °C, 20 °C, and 50 °C under exclusion of light. Storage at 50 °C induced rapid particle growth within 3 days. Dispersions stored at 20 °C displayed improved stability, though became solidified within 90 days. Increase in temperature causes a decrease in micro viscosity leading to destabilization. However, Compritol solid lipid nanoparticles stored at 88 °C in the dark were stable over the storage period of 3 years. Storing the dispersions at higher temperature leads to a reduction of the zeta potential faster than storing at a lower temperature. Thus, if solid lipid nanoparticles dispersions are not exposed to light and stored at lower temperatures, the zeta potential remains practically unchanged and the dispersions are stable. The energy input in the form of light and temperature changes the crystalline structure of the lipid. This crystal orientation can result in a change in Nernst potential and simultaneously zeta potential (Freitas and Muller 1998).

Freitas and Muller (1999a) reported that the recrystallization index has an impact on the long-term stability of aqueous solid lipid nanoparticles dispersions. In general, dispersions with higher crystallized lipid phase exhibited an increased particle growth. Depending upon the nature of lipid, recrystallization of the lipid (after solid lipid nanoparticles formation) takes place very quickly within minutes. However, it can be retarded up to weeks or months. In general, the recrystallization index of the solid lipid nanoparticles is below the crystallinity of the bulk material used for solid lipid nanoparticles production and increases with increasing storage time.

8.7 Toxicity and Status of Excipients

Toxicity and the status of excipients are principal issues for the use of a drug delivery system. To ensure the safety of a drug delivery system, there is a compulsion to carry out toxicity studies. This represents a major hindrance for product entrance in the pharmaceutical market for the use of patients. Topical and oral administration of solid lipid nanoparticles is certainly non-problematic about the excipients. For the topical purpose, all excipients can be used which are currently incorporated for the formulation of pharmaceutical and cosmetic ointments and creams. Similarly, for oral solid lipid nanoparticles formulations, all the lipids and surfactants used in traditional dosage forms such as tablets, pellets, and capsules can be utilized (must be approved from the Food and Drug Administration), until there are few solid lipid particles formulations available in the market for parenteral injection. Therefore, a toxicity study would be necessary. To formulate parenteral solid lipid nanoparticles especially intravenous preparations, intravenous-accepted surfactants can be used such as lecithin, Tween 80, poloxamer 188, sodium glycocholate, Span 85, etc. The good tolerability of solid lipid nanoparticles has been established in both in vitro and in vivo studies. In cell cultures, solid lipid nanoparticles were compared with polyester nanoparticles. At 0.5% of polyester nanoparticles, 100% of the cells died, while at 10% solid lipid nanoparticles in the cell suspension, the viability remained at around 80% (Maaûen et al. 1993). Good tolerability was also found when performing bolus injections into mice. The administered dose was 1.33 g lipid/kg body weight, and 6 bolus injections were performed. For cetyl palmitate, no acute toxicity and no increase in liver and spleen weight were observed.

8.8 Therapeutic Applications of Solid Lipid Nanoparticles

Solid lipid nanoparticles represent numerous therapeutic applications due to their unique characteristics such as lipophilic nature, composition, nontoxicity, compatibility with wide range of drugs, and surface modification properties. Figure 8.4 depicts the therapeutic attributes of solid lipid nanoparticles.

8.8.1 Topical Drug Delivery

A domain of big potential for solid lipid nanoparticles and with a short time-to-market entry is topical products based on the solid lipid nanoparticles technology. Solid lipid nanoparticles have been recognized as the next-generation drug delivery system after liposomes. Similar to liposomes, they are comprised of well-tolerated excipients, and due to their small particle size, they sustain similar adherent properties. Improved dermal absorption of drugs loaded to solid lipid nanoparticles can be achieved by improving adhesion, decreasing particle size, and finely dispersion of

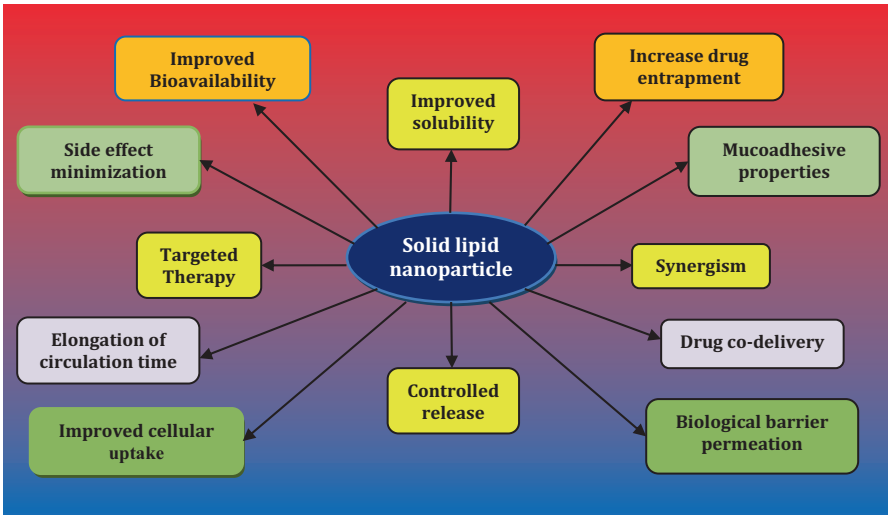

Fig. 8.4 Crucial attributes related to the application of solid lipid nanoparticles as drug reservoirs. Solid lipid nanoparticles offer numerous therapeutic applications through various routes of administration based on these features

drug within the lipid matrix of the carrier. Both these approaches facilitate solid lipid nanoparticles contact with the external skin layers. Solid lipid nanoparticles are composed of lipids, which are solid at ambient temperature as well as at skin surface temperature, which is 32 °C. Application of a solid lipid nanoparticles-loaded cream or gel to the skin surface induces structural changes of particle structure. Consequently, water evaporation results in a transformation of the lipid matrix to a more highly ordered structure leading to drug expulsion. Notable advantages of solid lipid nanoparticles are their solid state of the particle matrix and ability to shield chemically labile constituents against chemical breakdown and the possibility to modify drug release (Muller and Dingler 1998). Date and coworkers evaluated the potential of novel drug delivery systems including solid lipid nanoparticles to improve topical anti-acne treatment. They have reported that drug loading to carrier enhances penetration into the skin. Moreover, research investigations have proven that retinol incorporated into Compritol-based solid lipid nanoparticles released more rapidly and to a higher extent when compared with a nanoemulsion. This effect seems to result from a burst release from the solid particles following water evaporation on the skin surface and the change of lipid modification.

Solid lipid nanoparticles are potential formulations for topical delivery of antifungal drugs. Recently, El-Housiny et al. (2018) have formulated the well-known antifungal agent fluconazole-loaded solid lipid nanoparticles topical gel to enhance its efficacy for treatment of pityriasis versicolor, a superficial fungal infection caused by the proliferation of *Malassezia* species in the stratum corneum layer of skin. The clinical study has demonstrated the superior clinical efficacy of prepared

fluconazole-loaded solid lipid nanoparticles topical gel in contrast to marketed product Candistan in treating pityriasis versicolor. The results exhibited improved skin penetration due to enhanced contact between fluconazole and skin resulting from the large particle surface area and film formation. This permeation intensification of the fluconazole to the dermis layer of skin is very much needed as fungi hyphae (mycelium) can invade deeply through the epidermal layers; thus, fluconazole acts by preventing the production of vital elements in the fungal membrane such as ergosterol by inhibiting the fungal cytochrome P-450 enzyme (Nicky et al. 2008).

Similarly, Londhe and Save (2017) have developed a topical formulation of zaltoprofen-loaded solid lipid nanoparticles as a drug carrier system to be applied by topical application and to decrease side effects caused due to oral administration of zaltoprofen. Zaltoprofen, a nonsteroidal anti-inflammatory drug, possesses a novel anti-inflammatory mechanism that acts by inhibiting the bradykinin-induced responses by blocking bradykinin interaction with the bradykinin β2 receptor on dorsal root ganglion neurons (Tang et al. 2005). Zaltoprofen-loaded solid lipid nanoparticles were fabricated using solvent injection, emulsification, and evaporation method. The results of in vitro diffusion study showed that solid lipid nanoparticles-loaded gel exhibited sustained release as compared to plain drug-loaded hydrogel. In conclusion, the results confirm that solid lipid nanoparticles are efficient drug carrier system to encapsulate zaltoprofen and for sustained release of the drug. The phytoconstituent curcumin is enriched with therapeutic activity against various skin ailments. However, its clinical use encounters many hurdles associated to physicochemical and bioavailability characteristic such as curcumin has low water solubility in acidic pH conditions, rapid hydrolysis at an alkaline pH, and photosensitivity. Hence, inclusion and preservation of curcumin into conventional pharmaceutical dosage forms are challenging tasks. Moreover, curcumin has poor oral bioavailability (Lestari and Indrayanto 2014). Therefore, to resolve these issues, Goncaleg et al. (2017) developed cationic solid lipid nanoparticles. They have summarized that curcumin-loaded cationic solid lipid nanoparticles can directly enhance targeted drug delivery to diseased tissue, hence reducing adverse effects on normal cells. Consequently, the positive charge provided by these formulations favors drug targeting to cells in a deregulated apoptosis process, that have a high-density negative charge due to exposure to phosphatidylserine on the surface of cell membranes. Therefore, the present study validates the use of curcumin in topical treatment for skin disorders.

8.8.2 Oral Drug Delivery

Oral administration of solid lipid nanoparticles is feasible as an aqueous dispersion or alternatively after transforming into a conventional dosage form, such as tablets, pellets, capsules, or powders. Chemical and enzymatic barriers in the gastrointestinal tract hinder the oral delivery of many labile drugs. The gastrointestinal epithe-

lium also contributes to poor permeability for numerous bioactive molecules. Drugs with poor aqueous solubility have difficulty to dissolve in the gastrointestinal tract, resulting in low bioavailability. Nanomedicine presents an opportunity to improve the delivery efficiency of orally administered drugs. Solid lipid nanoparticles used for oral administration of drugs offer several advantages over conventional formulations, including increased solubility, enhanced stability, improved epithelium permeability and bioavailability, prolonged half-life, tissue targeting, and least side effects. In the lipid-based solid lipid nanoparticles, the principal mechanism of absorption is that the drugs are absorbed from the nanoformulations in the solid state. Further, digestion of oral nanoparticles containing glycerides begins in the stomach by gastric lipase. The habitual mixing of gastric fluid with amphiphilic products of lipid digestion results in the formation of a crude emulsion. The solid lipid nanoparticles are further assimilated in the intestinal fluid. The degradation products of lipids such as monoglycerides and fatty acids are able to improve intestinal drug transport by the production of mixed micelles with bile acids and the resulting uptake into the enterocytes. The large effective surface area by the inherent character of the nano-sized solid lipid nanoparticles can lead to an enhanced absorption rate (Lin et al. 2017).

Olmesartan is an antihypertensive drug with poor water solubility, high lipophilicity, and limited bioavailability. It is an angiotensin II receptor antagonist that blocks the binding of angiotensin II at the angiotensin I receptor in vascular smooth muscles, thus exerting antihypertensive effect. Therefore, in order to improve its oral bioavailability, Pandya et al. (2018) have developed solid lipid nanoparticles of olmesartan medoxomil using hot homogenization method. In vitro study of olmesartan-loaded solid lipid nanoparticle exhibited controlled release profile for at least 24 h. The rate and extent of drug diffusion were studied using dialysis sac, rat's stomach, and intestine tissues. Results obtained revealed that drug release from the solid lipid nanoparticles was significantly higher than suspension of olmesartan. In vivo pharmacokinetic study of olmesartan-loaded solid lipid nanoparticles revealed increased relative bioavailability by almost 2.3-folds compared to marketed formulation. These results suggest that loading of olmesartan in solid lipid nanoparticles enhances the bioavailability and therapeutic effect of drug. Hence, these solid lipid nanoparticles represent a great potential for a possible alternative to conventional oral formulation in the treatment of hypertension. Baek and Cho (2013) developed the surface-modified paclitaxel-filled solid lipid nanoparticles with hydroxypropyl-β-cyclodextrin. This hydroxypropyl-β-cyclodextrin has been recognized to solubilize the drugs and inhibit oxidation of lipids. The formulated solid lipid nanoparticles showed a paclitaxel encapsulation percentage of 71% with a mean size of 251 nm. The Caco-2 cell uptake of paclitaxel from solid lipid nanoparticles was 5.3-fold greater than the Taxol formulation.

Doxorubicin is an anthracycline antibiotic that can inhibit various malignant tumors. Doxorubicin act by inhibiting the progression of topoisomerase II, an enzyme that relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process

of replication. The major clinical limitations of doxorubicin are its serious cardiotoxicity and hepatotoxicity. To overcome these issues, Patro et al. (2013) investigated the oral bioavailability and toxicity of doxorubicin-loaded solid lipid nanoparticles with an emulsifier system of soy lecithin and poloxamer 188. The prepared solid lipid nanoparticles exhibited increased peak plasma concentration and reduced clearance when compared to free doxorubicin. The mean survival time of breast cancer-bearing rats increased from 34 days (positive control) to 47 days and 88 days in the free doxorubicin and solid lipid nanoparticles groups. Cardiac toxicity measured by the lactic dehydrogenase level was less in the solid lipid nanoparticles-treated group compared to the free control.

Carvedilol is an antihypertensive drug, acts by blocking α-1-receptor, and causes vasodilatation, and this inhibition leads to decreased peripheral vascular resistance and exerts the antihypertensive effect. Short half-life, first-pass metabolism, and low bioavailability are formulation barriers of carvedilol. To overcome the above-mentioned issues, El-Say and Hosny (2018) have developed carvedilol-loaded solid lipid nanoparticles by using hot homogenization followed by ultrasonication technique to provide controlled release and to enhance its bioavailability. The optimized solid lipid nanoparticle formulation was evaluated in vitro and in vivo for pharmacokinetic parameters on male New Zealand white rabbits. The result obtained showed that the prepared solid lipid nanoparticles prolonged the carvedilol release profile, maintained the concentration of drug in plasma for more than 23 hours, and significantly enhanced the oral bioavailability. Hence, it can be concluded that carvedilol-loaded solid lipid nanoparticles with improved biopharmaceutical features signify an effective formulation approach of nanomedicine in the management of cardiac diseases. Table 8.4 summarizes the different orally administered solid lipid nanoparticle formulations used to treat various ailments.

8.8.3 Pulmonary Drug Delivery

Till date, very few works have been done to explore the potential of solid lipid nanoparticles for pulmonary drug delivery, and very few pieces of literature have been promulgated in this domain. To illuminate the suitability of solid lipid nanoparticles as pulmonary drug delivery, aqueous solid lipid nanoparticles dispersions were nebulized with a Pari-Boy (nebulizer), the aerosol droplets were collected, and the size of particles were estimated. The results showed that particle size distributions of solid lipid nanoparticles before nebulization and after nebulization were nearly indistinguishable and only very little aggregation could be identified. Main advantages of drug release from solid lipid nanoparticles in the lung are controlled release of the drug, achievement of an extended release, and rapid degradation in comparison to polymeric materials. Along with that, solid lipid nanoparticles have been validated to possess high tolerability and facilitate targeting to lung macrophages. To sum up, there could be a huge potential waiting to be exploited (Lippacher et al. 2001). Systemic administration of antitubercular medicines can be compli-

Table 8.4 Formulations of orally administered solid lipid nanoparticles loaded with drugs and natural compounds against CNS-related disorders, tuberculosis, antiviral, and antimalarial

Active ingredients	Indication	Lipid type	Outcomes offered by solid lipid nanoparticles	References
Sumatriptan	Migraine	Tripalmitin	Improved AUC brain/AUC plasma ratio and photophobia	Hansraj et al. (2015)
Rizatriptan	Migraine	Precirol	Improved brain uptake and photophobia	Girotra and Singh (2017)
Sulpiride	Psychosis	Stearic acid and Dynasan 118	Increased gut permeability	Ibrahim et al. (2014)
Chrysin	Alzheimer's disease	Stearic acid	Improved memory loss	Vedagiri and Thangarajan (2016)
Isoniazid	Tuberculosis	Compritol 888	Increased bioavailability and less acute toxicity	Bhandari and Kaur (2013)
Lopinavir	Retrovirus	Stearic acid	Increased bioavailability	Negi et al. (2013)
Primaquine	Malaria	Stearic acid	Enhanced antimalarial efficacy	Omwoyo et al. (2014)
Arteether	Multidrug-resistant malaria	Monostearin	Increased bioavailability and half-life	Dwivedi et al. (2014)
Praziquantel	Schistosomiasis	Stearic acid	Enhanced parasite killing	de Souza et al. (2014)
Efavirenz	Retrovirus	Compritol 888 ATO	Increased lymphatic uptake and bioavailability	Makwana et al. (2015)
Venlafaxine	Major depressive disorder	Monostearin	Increased AUC in both plasma and brain	Zhou et al. (2015)

Compritol 888 ATO: mixture of mono-, di-, and triglycerides of behenic acid
Dynasan® 118: tristearin

cated by off-target toxicity to cells and tissues that are not infected by *Mycobacterium* tuberculosis. Delivery of antitubercular drugs via nanoparticles directly to the infected cells has the potential to enhance efficacy and reduce toxicity. To exploit this potential of nanoparticles and the development of methods for delivering antitubercular drugs directly to the lungs via the respiratory route, Gaspar et al. (2017) have developed rifabutin-containing solid lipid nanoparticles for pulmonary administration. Rifabutin is an antibiotic that inhibits DNA-dependent RNA polymerase activity in susceptible cells. In this research, rifabutin-loaded solid lipid nanoparticles were successfully encapsulated in mannitol and trehalose microspheres using a spray-drying method, which resulted in dry powders with appropriate features for pulmonary administration. This method enables researchers to overpass stability issues of liquid nanoparticle formulations as well as to reach beneath the lung following pulmonary. The in vivo biodistribution of rifabutin-loaded solid lipid nanoparticles demonstrated that the rifabutin distributed to the tested organs 15 and 30 min post pulmonary administration. Their antimycobacterial activity was also assessed in a murine model of infection with a *Mycobacterium* tuberculosis strain H37Rv resulting in an enhancement of activity against *Mycobacterium* tuberculosis infection compared to non-treated animals. Reduced growth index values were achieved in all studied organs for mice receiving rifabutin in microencapsulated glyceryl dibehenate solid lipid nanoparticles in comparison with the control group. Therefore, it has been proved from the results of study that these microencapsulated solid lipid nanoparticles are a novel strategy to expedite the pulmonary delivery of therapeutic antibiotics for the treatment of mycobacterial infections in the lung, contributing to improved chemotherapy of tuberculosis.

Budesonide is a potent nonhalogenated corticosteroid with high anti-inflammatory effects. A group of researchers has developed a solid lipid nanoparticles system to deliver budesonide to the lungs. Budesonide-loaded solid lipid nanoparticles were prepared by the emulsification-solvent diffusion method. The prepared solid lipid nanoparticles exhibited high entrapment efficiency of drug, particles of a suitable size range, and controlled release profile. Thereby, the outcome of the research demonstrated the potential use of solid lipid nanoparticles for the controlled release of budesonide used in the treatment of asthma (Emami et al. 2015). Nowadays, the intranasal route to bypass the blood-brain barrier is an emerging trend, as this route presents a novel, practical, simple, and noninvasive path to bypass the blood-brain barrier and diminish the systemic exposure. Several researchers have described solid lipid nanoparticles potential for intranasal administration of drugs.

Joshi et al. (2012) have developed solid lipid nanoparticles formulation of ondansetron, a serotonin (5-hydroxytryptamine) subtype (5HT3) receptor antagonist used in the management of chemotherapy-induced postoperative nausea and vomiting. Its absolute bioavailability is about 60 % due to its first-pass metabolism and the plasma half-life about 3–4 hours. This is evident from the literature that the drug uptake into the brain from the nasal mucosa mainly occurs via the olfactory pathway by which the drug partially travels from the nasal cavity to the CSF (cerebrospinal fluid) and/or brain tissue. The optimized formulation of this investigation was subjected to gamma scintigraphic study conducted on rabbits. Gamma scintigraphy

is a sophisticated technique in which the transit of a dosage form to the intended site can be noninvasively imaged in vivo via the careful introduction of an appropriate short-lived gamma-emitting radioisotope. Further, scintigraphic images obtained from experimental animals at 1, 2, 4, and 6 h after intranasal administration displayed a rapid accumulation of the radiolabeled drug in the brain, consequently distribution to various organs. In summing up, the results of the present study revealed that the intranasal administration of ondansetron-incorporated solid lipid nanoparticle formulation has the vital potential for targeting the central nervous system to achieve immediate onset of action.

Donepezil is a piperidine-based, reversible, and noncompetitive inhibitor of the enzyme acetylcholinesterase (AChE). It is the second drug approved by the Food and Drug Administration (FDA) for the treatment of mild to moderate dementia of Alzheimer's type (Zhiyong et al. 2006). Donepezil produces its therapeutic effect by increasing the concentration of acetylcholine through reversible inhibition of its hydrolysis by acetylcholinesterase, thus enhancing the cholinergic function. But due to hydrophilic nature of donepezil, its entry is restricted into the brain, and numerous side effects such as diarrhea, nausea, anorexia, and gastric bleeding are associated with frequent dosing of the conventional oral dosage form. As per the previous research findings, donepezil also exhibited hepatotoxicity and undergoes first-pass metabolism. Therefore, to overcome the abovementioned issues and to ensure the delivery of donepezil by non-oral route, Yasir et al. (2018) have developed solid lipid nanoparticles of donepezil. Solid lipid nanoparticles were prepared by solvent emulsification-diffusion technique using glyceryl as lipid and blend of Tween 80 and poloxamer 188 (1:1) as surfactant further evaluated for various in vitro and in vivo parameters. In vitro release study showed that optimized donepezil solid lipid nanoparticle formulation was more sustained as compared to donepezil solution. Pharmacokinetic and brain targeting studies in rodents exhibited a significantly high concentration of donepezil in the brain upon intranasal administration of donepezil-incorporated solid lipid nanoparticles compared to donepezil solution. The results of biodistribution studies were in accordance with the results of pharmacokinetic studies and establish brain targeting potential of developed formulations.

8.8.4 Parenteral Administration

Solid lipid nanoparticles can be administered through all parenteral routes appropriate for polymeric nanoparticles. This covers from intra-articular to subcutaneous and intravenous administration. Several researchers have developed solid lipid nanoparticles for intravenous administration. Bocca et al. (1998) produced stealth and non-stealth solid lipid nanoparticles and examined them in cultures of macrophages and after loaded them with paclitaxel in vivo. The intravenously administered solid lipid nanoparticles led to greater and extended plasma levels of paclitaxel. Surprisingly, both non-stealth and stealth solid lipid nanoparticles displayed a simi-

lar low uptake by the liver and the spleen macrophages, and a very impressive point was the increased uptake recognized in the brain. This study describes notably the potential of solid lipid nanoparticles to achieve prolonged drug plasma levels. Additionally, they have observed similar low uptake by the liver and spleen macrophages. This might be favored by a similar low-surface hydrophobicity of both types of particles, avoiding the absorption of any blood proteins mediating the uptake by liver and spleen macrophages. The uptake of the solid lipid nanoparticles by the brain might be explained by adsorption of a blood protein mediator.

Gemcitabine is a nucleoside analog used in chemotherapy of pancreatic, bladder, and breast cancer. The major constraint of gemcitabine is short half-life and severe side effects when administered intravenously (Li et al. 2009; Jia et al. 2010). These inherent drawbacks of parenteral gemcitabine administration paved the way for the development of an alternative drug delivery system. Nandini et al. (2015) developed gemcitabine-loaded solid lipid nanoparticles by double emulsification technique incorporating stearic acid as lipid, soy lecithin as surfactant, and sodium taurocholate as cosurfactant. The result of in vivo tissue distribution study of the optimized solid lipid formulation displayed an increase in cellular uptake by various organs over the free drug, where gemcitabine, after entering the cell, undergoes phosphorylation and acts by inhibiting DNA synthesis, thereby leading to cell death. Therefore, these lipid nanoparticles represent an effective colloidal carrier for enhanced cellular uptake of drug molecules and controlled release. Tumor formation is often associated with the development of defective and leaky blood vessels. Nanoparticles can pass through the leaky blood vessels into the tumor tissue and hence accumulate (Wong et al. 2006). To observe this phenomenon, Harivardhan Reddy et al. (2005) have developed etoposide-loaded solid lipid nanoparticles stabilized with sodium tauroglycocholate and phosphatidylcholine, and the outcome was astonishing. They affirmed that intravenous administration of etoposide-loaded solid lipid nanoparticles led to a higher etoposide accumulation in the tumor of lymphoma tumor-affected mice when compared to a solution of the compound. Etoposide produces its antitumor effect by inhibiting DNA topoisomerase II, thereby ultimately inhibiting DNA synthesis, and ultimately promotes apoptosis of the cancer cell. To sum up, etoposide-loaded solid lipid nanoparticles exhibited significant antitumor effect and delineate the concept of permeability of nanoformulation into the tumor tissue.

8.8.5 Improved Bioavailability and Biological Barrier Permeation Enhancement

Since last two decades, lipid-based nanotechnology drug delivery system has emerged as a foremost pharmaceutical approach to overcome the solubility and permeability issues associated with drugs having poor oral bioavailability. According to the Biopharmaceutical Classification System (BCS) for any drug, its oral bioavailability chiefly depends on its solubility behavior in the gastrointestinal fluid and permeability across the various biological membranes. Lipids are known to

enhance oral bioavailability of hydrophobic drugs in different ways like facilitating dissolution as a micellar solution and improving the lymphatic uptake. Lipid nanoparticles, namely, solid lipid nanoparticles, are attractive carriers for per oral delivery of drugs with poor oral bioavailability as they are composed of lipid excipients, which are cheap, easily available, and nontoxic. Moreover, the manufacturing technique is simple and readily scalable for large-scale production, provides controlled release of active components, and has no stability issue.

Numerous drugs have been incorporated into lipid nanoparticles with the purpose of improving their poor oral bioavailability. Paclitaxel, a potent naturally occurring anticancer agent, is a non-ionizable, lipophilic molecule with hydrophobic nature. Pandita et al. (2011) prepared paclitaxel-loaded solid lipid nanoparticles by a modified solvent injection method where stearylamine was incorporated as lipid and poloxamer 188 and lecithin were incorporated as surfactants. In vitro release study of paclitaxel-loaded solid lipid nanoparticles showed sustained release. In vivo pharmacokinetic evaluation conducted in mice showed that the solid lipid nanoparticles after oral administration significantly increase drug concentrations in plasma and tissues compared to the free paclitaxel solution. Additionally, both the rate and extent of absorption of drug-loaded solid lipid nanoparticles in systemic circulation were found to be greater in comparison to the solution. In another study, researchers developed wheat germ agglutinin (WGA)-conjugated solid lipid nanoparticles. The prepared nanoparticles significantly increased the oral bioavailability and lung targeting potential of paclitaxel due to bioadhesive property of the solid lipid nanoparticles and targeting specificity of the conjugated ligand (Pooja et al. 2016).

Ball et al. (2018) have investigated the delivery of siRNA (small interfering ribonucleic acid) via lipidoid (amphiphilic lipid-like molecules) solid lipid nanoparticles under the simulated stomach and intestinal conditions in vitro. Results of in vitro evaluation exhibited that lipid nanoparticles were able to shield the entrapped nucleic acid in simulated gastric conditions. Further, to evaluate the stability of the siRNA-loaded solid lipid nanoparticles, they were exposed to different concentrations of pepsin and bile salts, and it was observed that exposure to the concentration corresponding to the fed state had a significant effect on the stability of the nucleic acid than the fasted state concentration. Biodistribution studies performed in mice revealed that nucleic acid-loaded solid lipid nanoparticles were retained in the gastrointestinal tract for a minimum period of 8 hours and the nanoparticles were able to enter the epithelial cell lining of the colon and small intestine. Aforementioned study delineates that solid lipid nanoparticles can be preferably used for delivery of siRNA to intestinal epithelial cell.

Demirel et al. (2001) developed piribedil solid lipid nanoparticles and investigated rate and extent of the drug in the systemic circulation of rabbits after oral administration. Piribedil is a dopamine D2 agonist. It is used in the treatment of Parkinson disease, particularly for the alleviation of tremor. Results showed that bioavailability of piribedil enhanced more than twofold compared with standard drug piribedil. The nanometric sizes of solid lipid nanoparticles allow their effective crossing of biological barriers. It has been observed that solid lipid nanoparticles

move easily through the cell membrane, distribute throughout the cytosol, move among various cellular levels, and localize in the perinuclear region. Enhanced permeation through biological barriers may also result from other factors including solid lipid nanoparticles composition or administration route. Enhanced permeation of drug through the BBB (blood-brain barrier) has been observed for antibiotic-loaded solid lipid nanoparticles. Biodistribution studies performed by Kumar et al. (2014) showed a 3.15 and 11.0 times higher concentrations in the brain and blood of mice, respectively. They have demonstrated that intranasal administration of streptomycin sulfate-loaded solid lipid nanoparticles in comparison to free antibiotic, intranasal administration of solid lipid nanoparticles is proposed to bypass the organs of the reticuloendothelial system consequently, and their systemic availability and concentration across the blood-brain barrier (BBB) are expected to be higher in comparison to the poorly available free antibiotic.

Dhawan et al. (2010) formulated solid lipid nanoparticles of quercetin, a natural flavonoid with proved antioxidant activity, for intravenous administration in order to improve its permeation across the blood-brain barrier. The solid lipid nanoparticles of quercetin were formulated using Compritol as lipid and Tween 80 as surfactant through a microemulsification technique. The optimized formulation exhibited a particle size of less than 200 nm, 85.73% drug entrapment efficiency. In all the in vivo behavioral and biochemical tests, the rats treated with solid lipid nanoparticles-encapsulated quercetin showed markedly better memory retention comparable to test and pure quercetin-treated rats. Improved drug permeation was also observed across the blood-brain barrier after intravenous administration of quercetin-loaded solid lipid nanoparticles to rats as compared to other tested groups of rats. The studies demonstrated successful targeting of the potent natural antioxidant, quercetin, to the brain as a novel strategy having significant therapeutic potential to treat Alzheimer's disease.

8.8.6 Cosmetic Applications

It is a well-known fact that lifestyle factors and environmental factors such as sunbathing, smoking, and pollution stimulate skin aging. The common mechanism involved is the formation of free radicals. These free radicals ultimately lead to DNA damage and the formation of oxidized lipids and proteins. The cells in our tissues normally react with antioxidant molecules by an upregulated expression of antioxidant and detoxification enzymes. Therefore, in the search of effective antiaging cosmetic preparation and improving peptide delivery into the skin, Suter et al. (2016) developed heptapeptide-loaded solid lipid nanoparticles. In this study, the main formulation development challenge was to ensure the delivery of this hydrophilic peptide in a stabilized and encapsulated form to the target cytosol in skin cells. The solid lipid nanoparticles were synthesized by using hot high-pressure homogenization method, which combine advantages such as physical stability, protection of incorporated labile active molecule, and controlled release. To evaluate

multicellular protection against ultraviolet-induced stress, study with skin explants has been performed. The application of optimized heptapeptide-loaded solid lipid nanoparticles formulation on skin explants exhibited significant and dose-dependent protection against ultraviolet irradiation. In the clinical suction blister study, irradiation with UV light for two hours after the final product application led to a statistically meaningful increase of the 8-OhdG (8-hydroxy-2′-deoxyguanosine) concentration in the human epidermis. The skin treated with marketed formulation showed a statistically significant 20% decrease in DNA (deoxyribonucleic acid) damage compared to placebo. In conclusion, the results of the study suggested that solid lipid nanoparticles technology enabled peptide delivery into the skin and supporting it to perform protective functions.

Ultraviolet radiation induces loss of elasticity and leads to premature aging, skin burns, erythema, and induction of skin cancer. Solid lipid nanoparticles have been emerged as the novel carriers for cosmetics, particularly to agents, which protect from harmful ultraviolet radiation. Their small size ensures that they are in close contact with the stratum corneum, which increases the penetration of active ingredients through the skin. The crystalline cetyl palmitate solid lipid nanoparticles have the ability to reflect and scatter ultraviolet radiation on their own, thus leading to photoprotection without the need for molecular sunscreens. Introduction of sunscreens into solid lipid nanoparticles directs to a synergistic photoprotection. Photoprotection effect was increased significantly, after incorporation of the molecular sunscreen compound 2-hydroxy-4-methoxy benzophenone into the solid lipid nanoparticles dispersion. Titanium dioxide (an opacifier) can be added to solid lipid nanoparticles formulation as well, and solid lipid nanoparticles displayed a higher reflection of ultraviolet radiation compared to conventional emulsions. Incorporation of molecular sunscreens has not only an additive but also a synergistic effect on absorbing capacity. This has been proved that incorporation of molecular sunscreen oxybenzone in solid lipid nanoparticles decreased the rate of release compared with equally sized emulsions, by up to 50%. Penetration ability of the active substance into stratum corneum was estimated by tape-stripping method. It has been reported that the rate of release is completely dependent upon the type of formulation. In vivo study showed that oxybenzone released and penetrated into human skin more quickly and to a greater extent from the emulsion (Wissing and Muller 2001). A prolonged release is of interest for perfumes as well as for perfumes incorporated into cosmetic products. Wissing and coworkers found that solid lipid nanoparticles loaded with the perfume Allure exhibited a prolong release of the perfume from the solid lipid matrix of solid lipid nanoparticles. Comparing the release of the perfume from an emulsion and solid lipid nanoparticles, after 6 hours, 100% of the perfume released from the emulsion, but only 75% released from the solid lipid nanoparticles (Wissing et al. 2000). Similarly, Hommoss et al. studied the effect of changing the solid lipid of the perfume-loaded lipid nanoparticles on the release profile of the incorporated perfume (Hommoss and Müller 2006). Hence, it could be concluded that by selecting a solid lipid that can enclose the perfume in its solid matrix, a controlled release of perfume can be achieved.

8.8.7 Brain Drug Targeting

Two research groups introduced first-time solid lipid nanoparticles for brain drug targeting administration independently. Interestingly, pharmacokinetics investigation of two cytotoxic drugs, specifically camptothecin and doxorubicin, revealed drug accumulation into the brain when both drugs are loaded into solid lipid nanoparticles and administered by oral and intravenous route (Yang et al. 1999a, b; Zara et al. 1999). Both stealth and non-stealth stearic acid-labeled solid lipid nanoparticles were found in rat brain 20 minutes after intravenous administration. When the same kind of solid lipid nanoparticles was loaded with doxorubicin, significantly higher drug concentration was found in the brain of the animals treated with stealth solid lipid nanoparticles as compared to non-stealth solid lipid nanoparticles and doxorubicin solution. Remarkably, 30 min after administration of stealth solid lipid nanoparticles, the same doxorubicin concentration (10 μg/g of tissue) was found in the brain, heart, liver, lungs, and spleen. The overall plasma kinetics of stealth and non-stealth solid lipid nanoparticles provided to be significantly different from that of the doxorubicin solution.

Efavirenz is a non-nucleoside reverse transcriptase inhibitor used for the treatment of human immunodeficiency virus. Efavirenz is highly lipophilic in nature and undergoes extensive first-pass metabolism resulting in low bioavailability. To overcome these hindrances and facilitate brain targeting of drug with increased bioavailability, Gupta et al. (2017) have developed efavirenz-loaded solid lipid nanoparticles. The intranasal administration of the formulation showed 150 times more brain targeting efficiency and 70 times better absorption potential of the efavirenz-loaded solid lipid nanoparticles dispersion in comparison to the orally administered marketed formulation (capsule). Hence, it is logical to establish the conclusion that the developed formulation has significant potential for reducing the plasma viral levels with a low dose of efavirenz as well as targeting the brain.

Fatouh et al. (2017) developed agomelatine solid lipid nanoparticles to facilitate the targeted brain drug delivery. Agomelatine is a novel antidepressant drug; it undergoes an extensive first-pass metabolism leading to a diminished absolute bioavailability. Agomelatine exhibits serotonin 5-HT2 receptor antagonist activity and is transported directly from the nasal cavity into the brain by avoiding the blood-brain barrier through the olfactory region of the nasal epithelium and the trigeminal neural region, thus enhancing agomelatine brain bioavailability and achieving brain targeting (Haque et al. 2014). The pharmacokinetic study of the prepared solid lipid nanoparticles exhibited a significant increase in plasma peak concentration and indicates a notable contribution of the direct nose-to-brain pathway in the brain drug delivery. Table 8.5 summarizes the example of various solid lipid nanoparticle formulations used for brain targeting of drugs with different pharmaceutical approaches.

Table 8.5 The solid lipid nanoparticle formulations described to stabilize the molecules with physicochemical or biological instability, improve the bioavailability of drugs, and increase the permeability of drugs across the blood-brain barrier

Drug	Solid lipid nanoparticles functionalization	Particle size (nanometer)	Study addressing the blood-brain barrier	References
Solid lipid nanoparticles used to stabilize molecules with physicochemical or biological instability				
Camptothecin	Tween 80-coated solid lipid nanoparticles	<200	Biodistribution, uptake by brain capillary endothelial cells	Martins et al. (2012)
Camptothecin	Tween 80-coated solid lipid nanoparticles	130–160	Biodistribution	Martins et al. (2013)
Solid lipid nanoparticles used to improve the bioavailability of drugs that cross the blood-brain barrier				
Clozapine	Plain solid lipid nanoparticles	96.7–163.3	Biodistribution	Manjunath and Venkateswarlu (2005)
Noscapine	PEG-conjugated solid lipid nanoparticles	80.5	Biodistribution	Madan et al. (2013)
Piperine	Tween 80-coated solid lipid nanoparticles	312.0	Biodistribution	Yusuf et al. (2013)
Bromocriptine	Plain solid lipid nanoparticles	154.3–216.8	Parkinson disease model	Esposito et al. (2008)
Edelfosine	Tween 80-coated solid lipid nanoparticles	105.4–111.2	Biodistribution, glioma model	Mendoza et al. (2009)
Quercetin	Tween 80-coated solid lipid nanoparticles	152	Alzheimer's disease model	Dhawan et al. (2010)
Baclofen	Plain solid lipid nanoparticles	161.4	Biodistribution	Priano et al. (2011)
Solid lipid nanoparticles used to increase drug permeation through the blood-brain barrier				
Tobramycin	Plain solid lipid nanoparticles	80	Biodistribution	Bargoni et al. (2001)
Idarubicin	Plain solid lipid nanoparticles	85	Biodistribution	Harivardhan Reddy et al. (2005)
Curcumin	Tween 80-coated solid lipid nanoparticles	Narrow distribution	Biodistribution	Kakkar et al. (2011)
Ganciclovir	Borneol-modified solid lipid nanoparticles	113.7–142.5	Biodistribution	Ren et al. (2013)
Saquinavir	Monoclonal antibody-modified solid lipid nanoparticles	100–500	Human brain-micro vascular endothelial cells permeation	Kuo and Ko (2013)
Docetaxel, ketoconazole	Folic acid-grafted solid lipid nanoparticles	95.9	Biodistribution	Venishetty et al. (2013)

8.8.8 Carriers for Peptide and Protein Drugs

Protein and peptides are an important family of bioactive compounds that play a significant role in controlling various functions of the body. They offer many attractive attributes, but notwithstanding this, their physicochemical instability, rapid enzymatic degradation limits their oral and transdermal bioavailability. As discussed earlier in this chapter, solid lipid nanoparticles are nontoxic in comparison to polymeric nanoparticles because production techniques do not need to employ toxic organic solvents, which may also have a harmful effect on protein drugs. Therefore, solid lipid nanoparticles are more relevant carrier system to incorporate protein and peptides, especially for lipophilic proteins due to their hydrophilic nature, which readily dissolved the melted mixture. The first-time lysozyme was loaded in solid lipid nanoparticles as a model peptide drug. Since last two decades, researchers have frequently published encouraging results regarding the inclusion of several peptides and proteins in solid lipid particulate carriers (Table 8.6).

Insulin is a peptide hormone used in the treatment of diabetes mellitus. Repeated dosing frequency of insulin causes pain, allergic reactions, and insulin lipodystrophy around the injection site. It undergoes rapid enzymatic degradation in the harsh gastrointestinal environment. High molecular weight and lack of lipophilicity of insulin cause poor permeability across the intestinal epithelium. Due to such inherent detriments, the oral bioavailability of insulin is less than 1%. Therefore, in order to overcome physiologic and morphologic barriers to insulin absorption, Ansari et al. (2016) developed insulin-loaded solid lipid nanoparticles and administered it orally to overnight-fasted diabetic rats. Researchers found that insulin released from solid lipid nanoparticles in the intestinal lumen can be directly internalized, being the first responsible for the physiological effect. Later, it can be concluded that solid lipid nanoparticles undergo physiological degradation and the insulin enters into the blood circulation. It has been proved from earlier research that nanoparticles with hydrophobic surfaces, such as solid lipid nanoparticles, are taken up more frequently by the intestinal epithelium than those with hydrophilic surfaces (Eldridge et al. 1990). Therefore, it could be established that these two mechanisms have been responsible for the prolonged physiological effect of insulin after oral administration. The results showed that relative pharmacological bioavailability of insulin-loaded solid lipid nanoparticles was enhanced approximately five times of pure insulin solution. To sum up, solid lipid nanoparticles could protect insulin from degradation and enhance intestinal absorption.

8.8.9 Vaccine Adjuvants

To enhance the immune response of vaccine, adjuvants are incorporated in the vaccine preparation. In solid lipid nanoparticles, lipid components being in the solid state degrade gradually providing a prolonged exposure to the immune system.

Table 8.6 Protein and peptide molecules incorporated in solid lipid nanoparticles with different fabrication methods, drug release kinetics, and in vivo performance

Protein/peptide	Particulate system	Method of preparation	Incorporation efficiency	Cumulative release	Biological stability	References
Bovine serum albumin	Solid lipid nanoparticles	Adsorption onto solid lipid nanoparticles	Not available	Not available	Not available	Gualbert et al. (2003)
Calcitonin	Solid lipid nanoparticles	Solvent evaporation	90%	4%/6 h	Bioavailability proved in vivo	Erni et al. (2002)
Thymopentin	Solid lipid nanoparticles	Thermal-sensitive gel technology	61.97%	Not available	Not available	Yang et al. (2010)
Human serum albumin	Solid lipid nanoparticles	Adsorption onto solid lipid nanoparticles	12.4–32.4%	Not available	Not available	Cavalli et al. (1999)
Insulin	Solid lipid nanoparticles	Warm microemulsion (w/o/w)	78–84%	37.8%	Intact protein	Zhang et al. (2006)
Lysozyme	Solid lipid nanoparticles	HPH cold dispersion	43.2–59.2%	Not available	≈100% bioavailability	Almeida et al. (1997)
Thymopentin	Solid lipid nanoparticles	Warm microemulsion	5.2% or 1.7%	10%/6	Intact peptide	Morel et al. (1996)
LHRH	Solid lipid nanoparticles	Warm microemulsion	90%	≈10%/8 h	Not available	Morel et al. (1995)
Insulin	Solid lipid nanoparticles	Solvent evaporation	Not available	Not available	Intact protein	García-Fuentes et al. (2002)

LHRH luteinizing hormone-releasing hormone, *HPH* high-pressure homogenization

Degradation can be furthermore reduced using sterically stabilizing surfactants that hinder the anchoring of enzyme complexes. Advantages of the use of solid lipid nanoparticles compared to traditional adjuvants are their biodegradation and their good tolerability by the body. Mishra et al. (2010) investigated the potential of solid lipid nanoparticles as a carrier for hepatitis B surface antigen by surface modifications to improve their loading efficiency and the cellular uptake, using the subcutaneous route. Specific anti-hepatitis B surface antigen and immunoglobulin G antibody level in the serum was determined by ELISA (enzyme-linked immunosorbent assay). This immunization study was performed chiefly to investigate the immune adjuvant effect of the solid lipid nanoparticles in generation of the systemic immunity for hepatitis B. The results obtained demonstrated that subcutaneous immunization could be an efficient alternative approach for vaccination against hepatitis B. Particulate system like solid lipid nanoparticles may be a better carrier system for immunization because it serves as a signal for phagocytic cells and maintains less diffusivity and restricted movement. Additionally, sustained antibody titer suggests the better immunological potential of the system.

Penumarthi et al. (2017) synthesized solid lipid nanoparticles by a modified solvent emulsification method to study their potential to conjugate with plasmid DNA and deliver them in vitro to dendritic cells using eGFP as the reporter plasmid. The main highlight of the current study was the investigation of transfection efficiency in dendritic cells, which are the major antigen-presenting cells of the mammalian immune system. Additionally, to determine the fate of complexes upon uptake by dendritic cells, both these aspects paved the way to understand the efficiency of DNA-solid lipid nanoparticle complexes in targeting the immune system. The in vitro transcription results showed a significant increase in transfection rate compared to controls. Interestingly, the transfection rates of 1:10, 1:50, and 1:100 ratios of complexes were almost comparable to that of lipofectamine. Even though lipofectamine is an established transfection agent in vitro, researchers found that there is no evidence of its translation in vivo for biological applications. Based on these findings, the maximum possible safe concentration of DNA-solid lipid nanoparticles complexes for effective transfection is within the range of 1:10–1:100. The uptake studies showed the internalization of complexes by lysosomes and endosomal escape. These results confirmed the suitability of DNA-solid lipid nanoparticles complexes for application in biological systems.

8.8.10 Anticancer Therapy

Nanotechnology-based drug delivery system provides an exceptional platform for the delivery of anticancer agents in order to enhance their targeting ability and bioavailability. One of the main advantages of loading anticancer drugs into solid lipid nanoparticles is to enhance their cellular uptakes by bypassing the different multidrug-resistant mechanisms. Briefly, cancer cells operate a variety of mechanisms at the cellular level to diminish the toxicity of chemotherapeutic agents. These defense

mechanisms are seldom categorized as "cellular" drug resistance. The most notable one is the multidrug resistance (MDR) phenotype, which involves active efflux of a broad range of cytotoxic drug molecules out of the cytoplasm by membrane-bound transporters (Gieseler et al. 2003). Solid lipid nanoparticles not only protect drugs from rapid metabolism and clearance but also lessen the possible side effects of conventional anticancer drugs. Additionally, they also increase the efficacy and residence time of cytotoxic drugs. Cytotoxic anticancer agents are found to be heterogeneous. As there is a different category of compounds that act as anticancer agents, they differ from each other in molecular structure and physicochemical properties. Conventional formulations consisting of polymeric material may not bind to this distinct group of anticancer agents. Despite, solid lipid nanoparticles are versatile and have the ability to incorporate these cytotoxic drugs (Müller et al. 2000).

Resveratrol, an anticancer drug, is poorly soluble, undergoes fast presystemic metabolism, and results in low bioavailability. Wang et al. (2017) formulated the solid lipid nanoparticles of resveratrol to improve issue of solubility and low bioavailability. In the present study, the breast cancer cell line MDA-MB-231 was selected to explore the anticancer effect of resveratrol and solid lipid nanoparticles of resveratrol. The results of the study indicated that resveratrol and resveratrol-loaded solid lipid nanoparticles significantly inhibited cell proliferation in a dose-dependent manner. The enhanced anticancer effect of resveratrol-loaded solid lipid nanoparticles compared to free resveratrol may contribute to the lipophilic nature of the carrier, which facilitates the intracellular uptake. Bcl-2, a cell survival protein, is best known for its role to suppress apoptosis, whereas Bax proteins can induce cell apoptosis with two important markers to determine the anticancer effect of the prepared formulation. In this investigation, a significantly increased level of Bax and decreased level of Bcl-2 were found after the treatment of resveratrol-loaded solid lipid nanoparticles. Furthermore, the cell cycle in the G0/G1 phase significantly increased. They have also claimed the downregulation of cyclin D1 (a cell cycle-related protein) in the resveratrol solid lipid nanoparticles-treated MDA-MB-231 cells. This indicates the potential of the prepared formulation in cell cycle arrest in the G0/G1 phase via the mechanism of downregulation of cyclin D1 in cancer cells. Therefore, formulation development of resveratrol in polymeric or lipid-based delivery systems nanoparticles is a better approach to overcome these drawbacks of the drug.

Recently, Wang et al. (2018) developed solid lipid nanoparticles of curcumin to improve the therapeutic efficacy for breast cancer. Curcumin is well known for its therapeutic effects such as antibacterial, anti-inflammatory, antioxidant, and antitumor. But it exhibits instability and poor solubility. Therefore, in order to improve the cytotoxic effect and issue of instability and solubility, curcumin-containing solid lipid nanoparticles were fabricated. Researchers found that prepared formulation exhibited a significant cytotoxic effect against SKBR3 cells (a human breast cancer cell line). Results of in vitro cellular uptake study showed an enhanced uptake efficiency of the curcumin-loaded solid lipid nanoparticles by SKBR3 cells, and a higher rate of apoptosis was observed in cancer cells, compared to cells treated by the free drug. Moreover, findings of Western blot analysis suggested that developed

formulation could promote the ratio of Bax/Bcl-2 but decreased the expression of cyclin D1 and CDK4 (cyclin-dependent kinase 4, also known as cell division protein kinase 4, is an enzyme that in humans is encoded by the CDK4 gene). These results revealed that curcumin-loaded solid lipid nanoparticles could be a prominent chemotherapeutic formulation for breast cancer therapy.

Ellagic acid is a polyphenol known for its wide range of therapeutic applications, but the poor water solubility and low bioavailability have confined its therapeutic potential. In this note, Hajipour et al. (2018) developed solid lipid nanoparticle formulation of ellagic acid to enhance solubility and bioavailability including anticancer potential against prostate cancer cell line. Cytotoxicity of ellagic acid and ellagic acid-loaded solid lipid nanoparticles on prostate cancer cell line (PC3) was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the expressions of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax), which are involved in apoptosis, were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cytotoxicity evaluations showed that ellagic acid in solid lipid nanoparticles significantly inhibited prostate cancer cells grown in a low concentration compared to the ellagic acid. The results of qRT-PCR demonstrated the upregulation of Bax mRNA (messenger ribonucleic acid) level increases after cell treatment with ellagic acid-loaded solid lipid nanoparticles. The results of this study suggested that incorporation of ellagic acid in solid lipid nanoparticles enhances its efficacy than ellagic acid in upregulation of Bax and this regulation is probably one of the molecular mechanisms, through which ellagic acid exhibits apoptosis in prostate cancer cell line. To sum up, loading ellagic acid in solid lipid nanoparticles renders it more effective in the prevention of prostate cancer cell growth.

Battaglia et al. (2011) have investigated the cytotoxic potential of methotrexate-loaded solid lipid nanoparticles against MCF-7 and Mat B-III cell lines (a type of breast cancer line). The solid lipid nanoparticles were prepared by the coacervation method. The results of cytotoxic evaluation displayed increased cytotoxicity against MCF-7 and Mat B-III cell lines compared with free drug. Methotrexate belongs to antimetabolite category of anticancer drugs. Methotrexate acts by inhibiting dihydrofolate reductase enzyme, an enzyme that participates in the tetrahydrofolate synthesis. This tetrahydrofolate is required for DNA synthesis. Thus, methotrexate inhibits the synthesis of DNA. The in vivo preclinical study showed that after intravenous administration, higher blood levels were achieved and major drug accumulation within breast cancer-affected tissue was shown compared with drug solution alone.

Minelli et al. (2012) have explored solid lipid nanoparticles antiadhesive mechanism in cancer therapy. Minelli and coworkers have developed cholesteryl butyrate solid lipid nanoparticles with an aim to enhance the adhesion on the cancer cell since cancer cell adhesion to endothelium is essential for metastasis dissemination. Researchers have developed the cholesteryl butyrate solid lipid nanoparticles with cancer or endothelial cells, and adhesion was evaluated by computerized microimaging technology. Migration was detected by the scratch "wound healing" assay and the Boyden chamber invasion assay. Expression analysis of ERK (extracellular

regulatory kinase) and p38 MAPK (the mitogen-activated protein kinases) was performed by Western blot. The results obtained showed that solid lipid nanoparticles may act as an antimetastatic drug in cancer therapy.

8.8.11 Antitumor Drug Delivery

A tumor is usually associated with a defective, leaky vascular architecture because of the poorly regulated nature of tumor angiogenesis. In addition, the interstitial fluid within a tumor is usually partially drained by a poorly formed lymphatic system. As a result, submicron-sized particulate matter may preferentially extravasate into the tumor and be retained there. This phenomenon often pointed out as the "enhanced permeability and retention" (EPR) effect (Yang et al. 1999a, b). This EPR effect can be taken advantage of by a properly designed nanoparticle system such as solid lipid nanoparticles to achieve passive tumor targeting. By doing so, the aforementioned poor tissue specificity problem can be partly solved. Like other types of drug carrier used for cytotoxic drug delivery, such as polymeric systems and liposomes, solid lipid nanoparticles have the advantages of physical stability, protection of labile drugs from degradation, controlled release, and nontoxicity of formulation excipients. Some of the recent research investigations of antitumor drugs-loaded solid lipid nanoparticles have been discussed in this section.

Indirubin is a chemical compound of traditional Chinese medicine, considered as an anticancer agent, and it is generally produced as a by-product of bacterial metabolism. Indirubin exerts its antitumor effect by inhibition of glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinases (CDKs), and fibroblast growth factor receptor (FGF-R1) (Ding et al. 2010; Tokuyasu et al. 2018). However, the poor water solubility of indirubin has limited its use as an antitumor agent. Glioblastoma multiforme (GBM) is the most severe type of brain primary tumors. One of the major issues in targeting therapies of glioblastoma is the availability of the drug in tumor tissues. Therefore, in order to increase solubility and antitumor activity of indirubin, Rahiminejad et al. (2019) developed indirubin solid lipid nanoparticles, and their antitumor effects were evaluated on glioblastoma multiforme cell line. The solid lipid nanoparticles were prepared with cetyl palmitate and polysorbate 80 via high-pressure homogenization methods in hot mode. The prepared solid lipid nanoparticles revealed a small size (∼130nm) and high encapsulation efficiency (∼99%). Results of in vitro experiments showed good physical stability and controlled drug release pattern. In vitro cytotoxicity studies evaluated the comparative effect of indirubin-loaded solid lipid nanoparticles and free drug in a dose-dependent pattern to monitor drug delivery parameters such as dose reduction and the increased interval between the effective dose and toxic dose of the drug. The results demonstrated that the cytotoxic effect of indirubin-loaded solid lipid nanoparticles and free indirubin was pH-dependent and significantly increased in acidic conditions at all doses. Recently, Chuang et al. (2017) have developed pH-sensitive nanotechnology to target acidic pH environment of solid tumors. In conclusion, the

indirubin-loaded solid lipid nanoparticles reported in this study might be considered as an effective tool to improve the solubility, bioavailability, and controlled drug release including pH-dependent cytotoxicity potential of indirubin to treat heterogeneous tumors especially glioblastoma multiforme.

Several reports have demonstrated that nanoparticles in the systemic circulation are usually recognized as foreign substances and rapidly eliminated from the bloodstream by the reticuloendothelial system (RES) of macrophages, specifically by liver Kupffer cells. This phenomenon is problematic for the systemic delivery of drugs to non-reticuloendothelial system tumors or tissues, although reticuloendothelial system deposition is useful for treating tumors or diseases in which reticuloendothelial system-containing cells are the target (Joshi and Müller 2009; Shenoy et al. 2005). The rapid uptake of intravenously administered colloidal drug delivery system to animals by the reticuloendothelial system can be inhibited by saturating the reticuloendothelial system with blank colloidal carriers or blocking agents such as dextran sulfate or latex particles. Therefore, it could be assumed that blank, drug-free solid lipid nanoparticles might be effective as nontoxic, transient reticuloendothelial system-blocking agents, if they were composed of a biocompatible and biodegradable lipid matrix that could be promptly cleared from the blood into the reticuloendothelial system (Talegaonkar and Vyas 2005). In order to investigate these hypotheses, Jang et al. (2016) investigated the influence of preinjected blank solid lipid nanoparticles on in vivo tissue distribution, tumor targeting, pharmacokinetics, and antitumor activity of sterically stabilized camptothecin-loaded solid lipid nanoparticles.

Camptothecin is a potent anticancer drug that inhibits topoisomerase I during the S-phase of cell cycle. Camptothecin is hydrophobic in nature, and its active lactone form rapidly hydrolyzes to the inactive carboxylate form under physiological conditions, thus confining the delivery and clinical application of camptothecin in cancer therapy (Garcia et al. 2002). Camptothecin-loaded solid lipid nanoparticles composed of trilaurin-based lipid matrix containing poloxamer 188 and pegylated phospholipid as stabilizers were prepared by hot homogenization method and evaluated for in vitro characteristics and in vivo performance. The prepared solid lipid nanoparticles exhibited an in vitro long-term sustained-release pattern and effectively shield the camptothecin lactone form from hydrolysis under physiological conditions. Significant tumor targeting and tumor growth inhibition were observed after intravenous administration of camptothecin-loaded solid lipid nanoparticles to mice with subcutaneous transplants of CT26 carcinoma cells. In pharmacokinetic studies in rats, camptothecin-loaded solid lipid nanoparticles markedly enhanced plasma camptothecin level and prolonged blood circulation compared to free drug. Nonetheless, high uptake of camptothecin-loaded solid lipid nanoparticles by the reticuloendothelial system (RES)-rich tissues resulted in limited tumor targeting of camptothecin-loaded solid lipid nanoparticles and plasma camptothecin levels. Preinjection of blank solid lipid nanoparticles before administration of drug-loaded solid lipid nanoparticles to tumor-affected mice substantially reduced the accumulation of camptothecin-loaded solid lipid nanoparticles in RES organs. This led to noticeably enhanced tumor targeting, improved pharmacokinetic parameters, and

significant antitumor efficacy of camptothecin-loaded solid lipid nanoparticles (Jang et al. 2016). Therefore, camptothecin-loaded solid lipid nanoparticles with preinjected blank solid lipid nanoparticles could be a remarkable therapeutic strategy for safe and effective antitumor therapy. Anthracycline antitumor drugs are among the most powerful agents for antitumor activity. Doxorubicin hydrochloride (Adriamycin) is the most commonly used in the treatment of different carcinomas such as breast, lung, thyroid, and ovary. Despite this widespread use of anthracyclines in cancer therapy, they cause severe side effects also. To overcome the risk of anthracyclines-associated side effects, a doxorubicin prodrug, composed of a long lipophilic acyclic isoprenoid chain derived from squalene, has been investigated.

Stella et al. (2018) have attempted the feasibility of encapsulating the active lipophilic derivative squalene doxorubicin into solid lipid nanoparticles as well as the potential of solid lipid nanoparticles in releasing the active drug into tumor cells. The solid lipid nanoparticles were fabricated by using the technique of fatty acid coacervation and are stabilized by biodegradable polymers enriched with hydrophilic properties. This biodegradable polymers, once adsorbed onto the solid lipid nanoparticles surface, can prolong in vivo half-life by preventing opsonization. The prepared doxorubicin derivative-loaded solid lipid nanoparticles were spherically shaped, have a mean diameter of 300–400 nm, and showed 85% w/w drug entrapment efficiency. The effects on cell growth of drug-loaded solid lipid nanoparticles, free doxorubicin, and the prodrug have been examined using cytotoxicity and colony-forming assays in both human ovarian cancer line A2780 and A2780 res wild-type and doxorubicin-resistant cells.

Further assessments as to the treatment's ability to induce cell death by apoptosis have been carried out by analyzing the activation of caspase 3. Researchers also compared the ability of doxorubicin, squalene doxorubicin-loaded solid lipid nanoparticles, and empty squalene doxorubicin to inhibit the growth of human ovarian cancer cells. Briefly, cells were cultured either in the presence or the absence of titrated amounts of each sample for 72 h, and the number of viable cells was then assessed using the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay for assessing cell metabolic activity. The results showed a concentration-dependent effect, with an inhibition of 70–90% observed at the highest concentrations. The drug-loaded nanoparticles induce an inhibitory effect that is similar to that of free drug in the A2780 cell line. The levels of cell death, evaluated by the activation of caspase 3, in experimented cell lines exhibited that the squalene doxorubicin-loaded solid lipid nanoparticles formulation showed the highest efficacy, compared to cells treated with squalene doxorubicin at the same concentration. Squalene doxorubicin and drug-loaded solid lipid nanoparticles significantly increased caspase 3 activity in the A2780 res cell line, compared to doxorubicin-treated cells, at all the concentrations tested. However, the drug-loaded solid lipid nanoparticles formulation exerted a higher efficacy. The in vitro data demonstrated that the delivery of the squalenoyl-doxorubicin derivative by solid lipid nanoparticles increases its cytotoxic activity, as well as its apoptosis effect. This effect was particularly evident in doxorubicin-resistant cells. Empty squalene doxorubicin appeared to be more active than other hydrophobic doxorubi-

cin prodrugs, but its incorporation in solid lipid nanoparticles demonstrated improved antitumor activity and physical stability against resistant cell lines.

8.9 Future Perspective of Solid Lipid Nanoparticles

Since the last two decades of research in nanotechnology, solid lipid nanoparticles have emerged as the biggest arsenal of nanomedicine. But yet, there is a long way to discover the potential of this novel drug delivery system to ensure the safe and effective delivery of drugs to the target site with different routes of administration. In the case of sterile dosage forms, they will represent more feasibility for various drugs possess poor aqueous solubility, short half-life, and low chemical and biological stability.

The role of solid lipid nanoparticles to enhance bioavailability of many newly developed active pharmaceutical ingredients from the biopharmaceutical classification system (Classes II, III, and IV) is a major challenge. Further, they can resolve problems associated with unpleasant taste, irritation, and first-pass metabolism in the gastrointestinal tract. Solid lipid nanoparticles will represent new formulation avenue for transdermal delivery of various drugs as it is evident from published literature that skin penetration of drugs may be enhanced due to the better occlusion of the solid lipid nanoparticles compared to traditional topical formulations.

Pulmonary and nasal applications of solid lipid nanoparticles have been proved safe, and to improve bioavailability further, solid lipid nanoparticles will offer an alternative carrier system for anti-asthmatic agents and for drugs targeting the brain by the nasal route to overcome limitations like low blood/brain uptake. This mechanism laid the foundation in the treatment of various complex diseases related to the central nervous system and disorders like Parkinson's, Alzheimer's, multiple sclerosis, and others. The futuristic approach of solid lipid nanoparticles certainly will explore the emerging trend of gene therapy, vaccine delivery as adjuvants, improvement of the loading of the RNA and DNA, and decreasing the toxicity of the final formulation. However, solid lipid nanoparticles cannot be considered as a replacement to all drug delivery problems due to some drawbacks such as polymorphic modifications of the lipid matrix, physical instability (gelation, aggregation, drug expulsion), sterilization, and antimicrobial preservation could also be problematic for many of the formulations. Additionally, the interaction of solid lipid nanoparticles with their biological surroundings such as adsorption/desorption processes, enzymatic degradation, agglomeration, and interaction with endogenous lipid carrier systems is needed to be investigated to establish them ideal for nanomedicine. In the future, a better understanding of the colloidal state of the lipids as a result of the more sensitive and modern analytical techniques will help the researchers to overcome some of the constraints. This is a well-known fact that a drug delivery system is to be considered successful with its strong market presence in terms of patient and physician compliance. To establish a considerable place in the global pharmaceutical market for solid lipid nanoparticles, it is very much essential that

the pharmaceutical industries along with the academic fraternity should focus on research and development of solid lipid nanoparticles formulations. For diagnostics, drug delivery, scale-up production and to establish these formulations in the market as safe, effective, and economical drug delivery system.

8.10 Conclusion

There is a flourishing discussion about the inherent advantages, potential therapeutic applications, and limitations arising from the application of nanomaterial-based medicine in biological systems. Solid lipid nanoparticles can be favorably used as an alternative colloidal drug delivery system, and solid lipid nanoparticles formulation appears to be very promising as a carrier system for high molecular weight compounds such as peptides, proteins, or DNA. Moreover, these nanoparticles can be introduced into various pharmaceutical formulations including cosmetic preparations. Solid lipid nanoparticles offer the advantage of their feasible manufacturing in research laboratories and its subsequent technology transfer to large-scale production. By selecting suitable excipients such as lipids, triglycerides, along with the appropriate concentration of emulsifiers, the requirements like entrapment efficiency and controlled release can be achieved. Apart from these significant attributes, it is expected to recognize the risk resulting from individual exposure to solid lipid nanoparticles. Furthermore, it is remarkably relevant to explain the interactions between solid lipid nanoparticles and their biological environment. Additionally, the great trial remains to avoid the dose dumping or burst effect of drugs incorporated in solid lipid nanoparticles. In conclusion, solid lipid nanoparticles represent a promising drug delivery system with vast technical and therapeutic potential. In conclusion, solid lipid nanoparticles are promising drug delivery system due to the nontoxicity aspect and a variety of drugs-loading capacity together with the advantages of delivery of drugs through all administration routes.

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Chapter 9
Nanomedicine: Diagnosis, Treatment, and Potential Prospects

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Abstract Treatment of diseases using conventional drugs is often limited by their low bioavailability, short circulation half-lives, poor solubility, and nonspecificity which results in high-dosage requirements. The high dosage of drug molecules results in higher toxicity, increasing the side effects of the conventional drugs used for treatment of diseases. Nanomedicine is the use of nanotechnology for healthcare with clinical applications ranging from disease diagnosis to formulation of carriers for drug and gene delivery applications. Use of nanotechnology-based delivery vehicles, such as nanoparticles, nanocapsules, micelles, or dendrimers, has emerged as a promising strategy to deliver conventional drugs, recombinant proteins, vaccines, and, more recently, genetic material by addressing the problems related to poor solubility, high toxicity, nonspecific delivery, in vivo degradation, and short circulation half-lives of the conventional drugs, which often limits optimal dosage at the target site. The rapidly growing nanomedicine industry not only caters to the treatment of various diseases including cancer, pain, asthma, multiple sclerosis, and kidney diseases but also helps in differentiating normal and diseased cells. Metallic, polymeric, semiconductor, and magnetic nanoparticles have been employed in engineering nanostructures that are increasingly being employed for disease diagnosis. While the unique optical, magnetic, and size-dependent properties of nanoparticles make them suitable candidates for disease diagnosis, their ability to undergo surface modification with polymers, antibodies, or aptamers helps in increasing their circulation time and reduces their potential toxicity. Conjugation of these nanoparticles with aptamers has been utilized for development of sensors with fluorescence, optical, and electrochemical detection signals which are sensitive, highly specific, reusable, and label-free. Nanostructures have improved medical diagnosis by providing inexpensive, reproducible, sensitive, and highly specific methods for disease diagnosis either in terms of sensors or as imaging agents. Nanomedicine not only includes the fields of therapeutics and diagnostics but also involves development of implantable materials and devices. Despite the innumerable advantages of nanostructures in the field of nanomedicine, only a handful of products have been able to reach the market due to several disadvantages that these magic bullets are associated with including toxicity of the said materials. However, maintenance of a balance between the advantages and disadvantages would definitely open up avenues for personalized medicine through therapeutics, diagnostics, and theranostics. The present chapter discusses the current state-of-the-art materials used in nanomedicine for disease diagnosis or treatment, problems associated with them, and future prospects of nanomedicine toward personalized medicine.

Keywords Nanobiotechnology · Nanodiagnostics · Nanoparticles · Aptamers · Personalized medicine

List of Abbreviations

ATP	adenosine triphosphate
bp	base pairs
DNA	deoxyribonucleic acid
FRET	Fluorescence Resonance Energy Transfer
H_2O_2	hydrogen peroxide
HIV	human immunodeficiency virus
IFN-γ	interferon-γ
PBCA	poly(butyl cyanoacrylate)
PDGF	platelet-derived growth factor
PLGA	poly-(lactic-co-glycolic) acid
QD	quantum dot
RNA	ribonucleic acid
SERS	surface-enhanced Raman scattering
VEGF	vascular endothelial growth factor

9.1 Introduction

Nanomedicine is the use of nanotechnology for healthcare with clinical applications ranging from disease diagnosis to formulation of carriers for drug and gene delivery applications. It is often regarded as the use of unique nanoscale properties which includes transition in physicochemical properties and physiological interactions of the nanostructured materials, i.e., structures with at least one dimension up to 300 nm, for human health. Nanostructures have a greater surface area to volume ratio which increases their carrying capacity and possibility of surface modifications. They exhibit unique size-dependent properties which are fairly distinct from those of bulk material, for example, 20 nm gold nanoparticles exhibit red color which changes to blue on increasing their size. The unique optical properties of nanoparticles make them suitable candidates for optical imaging. The use of nanostructures as drug carriers offers several advantages including higher stability, ability to incorporate both hydrophobic and hydrophilic drugs, and ability of controlled drug release. Moreover, nanostructures can be administered through various routes including inhalation, oral, intravenous, or intramuscular, reducing the intrusions and hence increasing patient compliance. The unique advantages of nanostructures including targeted delivery of drug, reduced side effects of free drug molecules, increased bioavailability, smaller and highly sensitive diagnostic tools along with reduced degree of invasiveness have resulted in a new field of medicine, "nanomedicine."

The first nanomedicine which was a liposomal formulation of anticancer drug doxorubicin was introduced in the year 1995, and since then, about 50 other nanostructure-based drugs have entered clinical practice (Min et al. 2015). A plethora of nanostructures including nanoparticles, nanocapsules, liposomes, micelles,

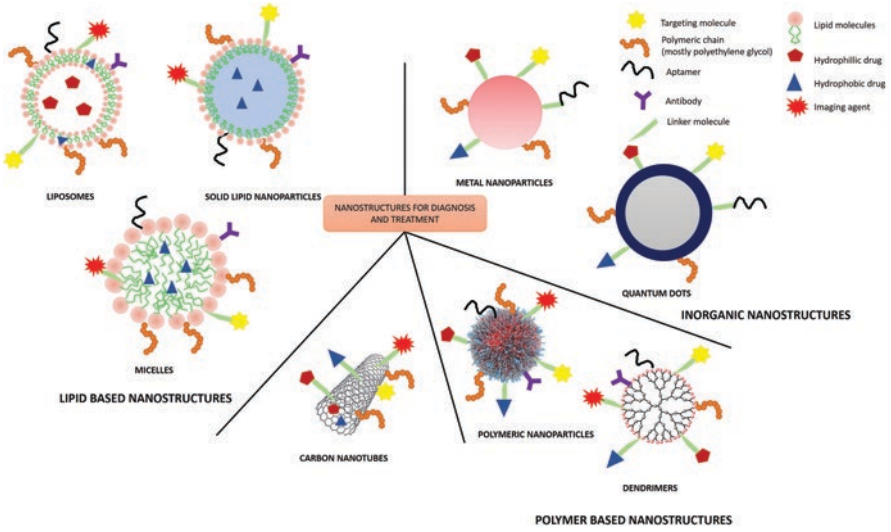


Fig. 9.1 Different nanostructures used for disease diagnosis and treatment

dendrimers, and quantum dots are being used as nanomedicines for the diagnosis and treatment of a wide variety of diseases (Fig. 9.1) including cancer, infectious diseases, multiple sclerosis, chronic pain, asthma, and emphysema (Hobbs et al. 1998). Nanomaterials are being used not only for drug and gene delivery applications but also for detection of pathogens (Edelstein et al. 2000), for tissue engineering (Ma et al. 2003; de la Isla et al. 2003), as contrast agents (Weissleder et al. 1990), for tumor destruction (Shinkai et al. 1999), and also as fluorescent biological labels (Bruchez et al. 1998; Chan and Nie 1998). According to a recent review on nanomedicine, by 2013, about 247 nanomedicine products were approved or were in various stages of clinical study (Etheridge et al. 2013). Nanomedicine has become a vast industry in a small span of just two decades due to its ability to address several issues related to poor solubility, high toxicity, nonspecific delivery, in vivo degradation, and short circulation half-lives of the conventional drugs, which often limits optimal dosage at the target site (Gelderblom et al. 2001). Nanostructures used for diagnosis due to their distinct optical, magnetic, and structural properties offer several advantages over the traditional diagnosis methods including but not limited to minimal invasiveness, simplicity, low-detection limits, rapid analysis at room temperature, and capability of in situ analysis (Jyoti and Tomar 2017).

Current application of nanostructures in the nanomedicine arena includes development of (a) implantable materials for tissue engineering including repair and replacement, (b) implantable devices including implantable sensors and surgical aids, (c) diagnostic tools for disease detection, and (d) biopharmaceuticals for drug and gene delivery (Agrawal 2016). A wide variety of nanomedicines are available commercially or are in clinical trials to treat diseases including breast cancer, non-small cell lung cancer, pancreatic cancer, ovarian cancer, and multiple myeloma as

well as diagnostic tools to detect disease-causing pathogens or to locate the tumor location (Agrawal 2016).

Nanostructures have presented themselves as promising nano-therapeutic agents, i.e., a combination of diagnosis and therapy. While the optical and magnetic properties of nanostructures make them great imaging agents, simultaneous drug loading in these nanostructures could help cure the disease at the same time. Nanotheranostics open up new avenues for personalized medicine. While an imaging agent can assist localization in the diseased area, its amalgamation with a therapeutic drug would help in treatment of the disease. The imaging nanostructure could also be utilized to monitor the effectiveness of the therapy in being site specific while predicting the side effects (accumulation in healthy cells) at the same time. Despite the promising potential of nanostructures in diagnosis and treatment of diseases, the technical developments in nanomedicine raise concerns related to the safety of the said materials, making toxicity of nanomaterials a major concern for their future developments. Moreover, the effects of these nanostructures on the biochemical pathways are yet unknown.

The current chapter discusses the application of different nanostructures in the field of nanomedicine for diagnostic as well as therapeutic purposes, followed by a brief overview of the drawbacks that these magic bullets face and the future directions to expand the nanomedicine market.

9.2 Nanostructures for Disease Diagnosis

A biosensor is a chemical sensor which comprises of a biological recognition element that recognizes the analyte and transducer which transmits the signal. Different biological interactions, e.g., antigen-antibody or antigen-aptamer, are utilized to generate signals such as optical, electrochemical, thermal, or piezoelectric that help in the detection of different disease-causing agents such as bacteria or virus. Nanostructures have high surface area per unit volume which locates all the constituent atoms near the surface leading to different physicochemical properties at nanoscale compared to the bulk solid. They have high electrical conductivity, better mechanical shock-bearing ability, show piezoelectric effect and color depending on the size of the nanoparticles. Features of different nanostructures like high electrical conductivity, colorimetric properties, and strong mechanical strength allow them to be suitable for conjugation with aptamers and antibodies in biosensing applications. Nanotubes (e.g., carbon nanotubes), nano-wires, nanorods, metallic nanoparticles (e.g., gold), quantum dots, and thin films made up of nanocrystalline matter are some widely used nanostructures for nano biosensing applications.

The emerging synergy between nanotechnology and biosensors has been utilized over the past few years due to their potential to recognize threat agents in real time and also perform their detection with extremely high sensitivity and selectivity. Nanostructures-based sensors help in rapid, sensitive, easy, and cost-effective detection of different biological molecules including disease-causing pathogens, proteins

expressed in different cancer cells, blood analytes such as glucose, cholesterol, uric acid, and albumin along with distinguishing normal versus cancer cells (Manoharan et al. 2018). Such sensors either use antigen-antibody interactions or functional nucleic acids as bio-recognition elements. Functional nucleic acids including aptamers, DNAzymes (catalytic DNA that can catalyze biochemical reaction in the presence of cofactors), and Aptazymes (a combination of aptamer and DNAzyme) (Li and Lu 2000; Jhaveri et al. 2000; Yi 2002; Navani and Li 2006), due to ability of in vitro production and higher selectivity are now rivaling the antibodies (Jayasena 1999). The following section discusses different nanostructures including metallic, polymeric, or magnetic nanoparticles, quantum dots, and aptamer-nanoparticle conjugates used for molecular diagnostics.

9.2.1 Metallic Nanoparticles for Diagnostics

The most widely used nanostructures for disease diagnosis are metallic nanoparticles made up of gold, silver, or metal oxides such as titanium dioxide. Gold and silver nanoparticles exhibit surface plasmon resonance which results in sharp, intense absorption band in the visible range, making them suitable candidates for development of optical nanosensors. Moreover, the properties exhibited by these nanoparticles can be tailored by controlling their size and structure. Gold and silver nanoparticles detect different disease-causing analytes, such as antigens, nucleic acids, or aptamers, based on interparticle distance. Reduction in the interparticle distance results in aggregation of particles which can be detected by a visible color change mainly red to purple or blue in case of gold nanoparticles (Fig. 9.2) and yellow to brown for silver nanoparticles, making them suitable candidates for visible optical detection (Boisselier and Astruc 2009). These particles are often surface modified to increase circulation time (generally with polyethylene glycol) and achieve targeted diagnosis (with antibodies or aptamers).

Surface-modified gold nanoparticles have been employed for detection of *Mycobacterium tuberculosis* and *Staphylococcus aureus* based on the aggregation principle. In presence of the microorganisms bound to the particles, they are unable to come together and aggregate (no color change), whereas the absence of microorganism results in visible aggregation in the form of color change on addition of sodium chloride (NaCl) (Baptista et al. 2006; Huang 2007). Gold nanorods, surface modified with polyethylene glycol and covalently attached to monoclonal antibody 'Herceptin' for targeting breast cancer cells, have demonstrated their potential in vitro as well as in vivo in tumor-bearing nude mice (Eghtedari et al. 2009). The most common use of metallic nanoparticles is for detection of hydrogen peroxide (H_2O_2), the most extensively studied reactive oxygen species in medicine, overproduction of which is associated with many diseases including cardiovascular diseases, diabetes, neurodegeneration, cancer, and aging. Recent advances in use of metallic nanoparticles have focused on development of bimetallic sensors. Zhang et al. (2016) combined the advantages of nano gold and silver to prepare core-shell

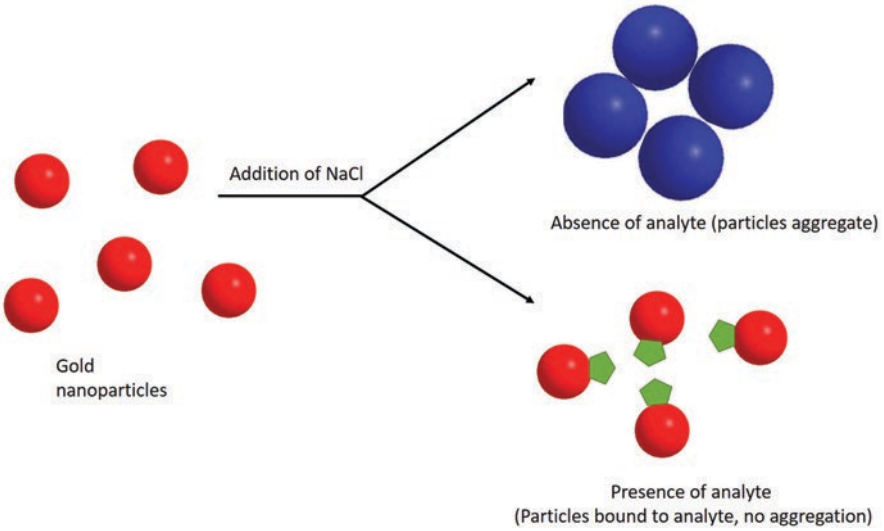


Fig. 9.2 Visible colorimetric sensing using gold nanoparticles. [Addition of NaCl results in aggregation (color change to blue or purple) of nanoparticles in absence of any analyte. However, presence of analyte increases the interparticle distance and hence prevents aggregation (red color)]

nanoparticles for detection of H_2O_2 in human fluids. The developed nanosensor exhibited greater stability provided by nano silver and higher surface plasmon effect displayed by nano gold. Gold nanoparticles exhibit a surface plasmon peak at 520 nm (wine red), whereas the developed bimetallic sensor exhibits a surface plasmon peak at 375 nm (orange). In the presence of H_2O_2 and glucose or cholesterol, a redox reaction results in reduction of silver. As a consequence, only gold nanoparticles are left in the solution, shifting the absorbance peak to 520 nm (wine red). The shift in peak can then be quantified to determine the concentration of H_2O_2 or cholesterol in the fluid being tested (Zhang et al. 2016). Other bimetallic sensors being explored for sensing of peroxide, glucose, and cholesterol include Au/Pt (Che et al. 2009; Yanyan et al. 2011), Pt/Pd, and TiO_2/graphene-supported Pt/Pd nanocomposites (Cao et al. 2013; Safavi and Farjami 2011).

Though metallic nanoparticles constitute the major market in disease diagnosis nanostructures made from polymers, magnetic material and semiconductors (quantum dots) discussed below are also being increasingly employed for effective disease diagnosis.

9.2.2 Polymeric Nanoparticles for Diagnostics

Synthesis of nanostructures using polymers such as polypyrrole and polyaniline for different sensing applications has recently gained attention due to their greater biocompatibility and biodegradability. Conducting properties of polypyrrole, carboxyl-

ated polypyrrole, polyaniline, and poly (3, 4-ethylenedioxythiophene) were employed by Park et al. (Park et al. 2016) and Song et al. (Song et al. 2013) to develop bioelectronic-based nanosensor for detection of hydrogen peroxide (H_2O_2). Hydrogen peroxide is a reactive oxygen species which is essential for cell growth and is thus related to several diseases including cancer and Alzheimer's. Polypyrrole-coated silver nanostrips integrated in an electrochemical sensor have also been developed for catalytic detection of H_2O_2. The enlarged surface area of silver nanostructures has been reported to facilitate rapid sensing (response time less than 5 s) and reducing minimum detection limit (Mahmoudian et al. 2014). Glucose sensing, important for monitoring diabetes, has been done using polymeric nanoparticles by employing a hybrid nanostructure entrapping glucose oxidase and gold nanoparticles in polyaniline layers. Gold nanoparticles were reported to assist polyaniline layer formation and the developed biosensor consisting of glucose oxidase as catalyst resulted in formation of hydrogen peroxide which was used as an indicator for presence of glucose (Mazeiko et al. 2013).

9.2.3 Magnetic Nanoparticles for Diagnostics

Magnetic nanoparticles are being used as a versatile diagnostic tool in nanomedicine. They are attached to antibodies or aptamers specific to the target molecule or microorganism, which on binding to specific targets result in magnetic signals that can be detected in the presence of magnetic field using a sensitive magnetometer. Magnetite, iron, nickel, and cobalt nanoparticles possess superparamagnetic properties at nanoscale, making them suitable candidates for being used as diagnostic tools. However, these nanoparticles are easily oxidized and tend to aggregate.

Prevention of oxidation and aggregation of magnetic nanoparticles has been achieved by constructing nanocomposites employing polymers on the outer layers. Iron nanoparticles of 15–20 nm have been embedded in beads of styrene and glycidyl methacrylate, resulting in 100–200 nm nanocomposites which are stable, exhibit a zeta potential of −58.4 mV, and show superparamagnetic properties that have been used for tracking cells and also for calcium sensing. Such nanoparticles at a size range of 2–3 nm have been used to diagnose undetectable lymph nodes (Atanasijevic et al. 2006). Polymer coatings also prevent nonspecific protein adsorption, an important requisite to biosensing (Jain 2007). Dextran-coated iron oxide nanoparticles have also been used to enhance visualization of intracranial tumors in magnetic resonance imaging. CellTracks are commercially available ferrofluids, i.e., a magnetic core surrounded by a polymer layer which has antibodies attached for capturing cells. The conjugated antibodies bind to the antigen on target cells. This commercially available ferrofluid has been used to detect circulating cancer cells and also for isolation of bacteria, making it easier to manage patients with serious infections (Manoharan et al. 2018). The toxicity of magnetic nanoparticles is

determined by the constituents of the particle including the magnetic core (e.g., magnetite, iron, nickel, and cobalt), the outer polymeric shell, along with the shape and size of the particle.

9.2.4 Quantum Dots for Diagnostics

Quantum dots or semiconductor nanoparticles have unique optical properties including simple, broad excitation range with a very narrow or sharp emission with the emitted wavelength being dependent on size and composition of quantum dots. These molecules exhibit size-dependent properties because of the presence of "bandgap." Bandgap is defined as the difference in the energy levels of the valence band, the primary residence of electrons, and the conduction band, the energy level to which the electrons reach after excitation. The movement of electron from conduction band to valence band when the excitation is stopped results in release of energy in the form of light. The high sensitivity, simple instrumentation, and higher photostability in comparison to organic or inorganic fluorescent dyes make them suitable for various diagnostic applications including tagging viruses and cancer cells. They can also be easily attached to cells, proteins, and nucleic acids making them powerful tagging agents. The availability of quantum dots in red and infrared colors enables whole blood analysis.

Carbohydrate-encapsulated quantum dots have been successfully employed in imaging of cancer. Immunofluorescent labeling of breast cancer marker Her2 has been achieved using polyacrylate-coated quantum dots that are covalently linked to antibodies (Jain 2007). Cell surface proteins of respiratory syncytial virus have been detected using quantum dots linked to antibodies (Bentzen et al. 2005). Dual color quantum dots, capable of excitation with a single light source, have also been developed for detection of respiratory syncytial virus within few hours. Respiratory syncytial virus while infecting lungs leaves its coat containing F and G proteins on the cell's surface. Antibody-linked quantum dots when come in contact with viral particles or infected cells stick to their surface assisting their diagnosis during the course of infection itself. However, the most commonly used quantum dots made of CdSe (cadmium selenide)-ZnS (zinc sulfide) release potentially toxic cadmium and zinc ions into the cells. The toxicity of such quantum dots can be prevented by capping them with zinc oxide which prevents Cd^{2+} formation on exposure to air (Jain 2007).

The synergy between metallic, polymeric, semiconductor, and magnetic nanoparticles has contributed in development of several nanomedicines for disease diagnosis. While the unique optical, magnetic, and size-dependent properties of nanoparticles make them suitable candidates for disease diagnosis, their ability to undergo surface modification with polymers, antibodies, or aptamers helps in increasing their circulation time and reduces their potential toxicity. Conjugation of these nanoparticles with aptamers has helped in development of more robust diagnostic tools which have been discussed in the following section.

9.2.5 Aptamer Nanoparticle Conjugates for Diagnostics

The current decade witnessed many advancements in aptamer-nanoparticle conjugates for diagnostic applications (Sharma et al. 2017; Dhiman et al. 2017; Sharma et al. 2016; Sharma and Shukla 2014). Aptamers modified with nanomaterials have great potential to be used in clinics for diagnostics purposes (Kalra et al. 2018; Kaur et al. 2018; Chopra et al. 2014). The conjugate can bind with a broad range of diverse targets ranging from small molecules and proteins to intact viruses and whole cells. Aptamers have been selected for small molecules like cocaine, aspartame, growth factors, toxins, peptides, viral proteins, and bacterial cells. Based on the transduction principle, the diagnostic and imaging applications can be further categorized into electrochemical, colorimetric, fluorescence, or magnetism based methods outlined in the following subsections.

Fluorescence-Based Sensors

The unique optical properties of quantum dots or semiconductor nanoparticles make them suitable for use in conjugation with aptamers for diagnostic and imaging applications. The first reported use of quantum dots in conjugation with aptamer was done by Levy et al. (Levy et al. 2005). In this work, they detected thrombin using "Fluorescence Resonance Energy Transfer (FRET)" principles (Fig. 9.3) (Lee et al. 2010). Thrombin aptamers were functionalized on quantum dots, and the complementary nucleotide strand had a quencher at the end. In the presence of thrombin in the test sample, the single-strand aptamer bound to thrombin and the complementary DNA-containing quencher were released that lead to fluorescence of quantum dot (Levy et al. 2005). In advancement of the above method, Choi et al. (2006) modified the sensor where interaction of thrombin with aptamers brought them close to quantum dots that resulted in selective quenching of fluorescence due to the charge transfer occurring from thrombin to the quantum dots with a limit of detection of 1 mM (Choi et al. 2006).

Meng et al. (Meng et al. 2016) devised a FRET-based biosensor for sensitive determination of adenosine triphosphate (ATP) using aptamers. In this setup, 5′-carboxyfluorescein (5′-FAM), gold nanoparticles were used as energy donor and acceptor, respectively, and were conjugated with complementary single-stranded DNA. This aptasensor was developed by hybridization between the complementary DNA strands of both donor and acceptor molecules. The setup could detect ATP in the linear range of 0.1–100 μM with limit of detection 15.2 nM. In the presence of specific ATP as the target, the FRET frequency of this detection system was gradually increased while nonspecific targets like uridine. Cytidine and guanosine triphosphates had no such significant changes in frequency (Meng et al. 2016). In order to reduce background fluorescence, enhance regeneration and long-term storage, the fluorophore-labeled DNA could be immobilized on a solid surface.

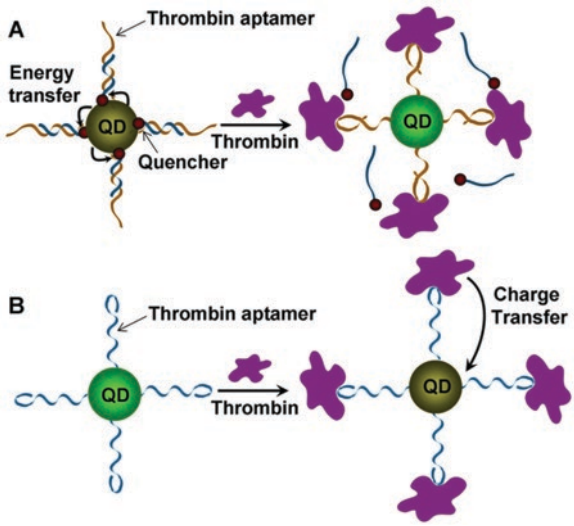


Fig. 9.3 Aptamer-quantum dot-based fluorescent sensors. (**a**) Thrombin aptamer is conjugated onto quantum dot and hybridized to a complementary DNA with a quencher. Fluorescence of quantum dot is quenched due to energy transfer from the quantum dot to the quencher. The complementary DNA containing the quencher can be released after introduction of thrombin, inducing recovery of the fluorescence from quantum dot. (**b**) Thrombin aptamers are conjugated to quantum dots. The fluorescence of quantum dots can be quenched as thrombin binds to aptamer due to the charge transfer from thrombin to quantum dot. [Adapted from Lee, Yigit (Lee et al. 2010) with permission from Elsevier]. (QD-quantum dot)

While quantum dots conjugation with aptamers has been used for fluorescence based sensors, metallic nanoparticles have been conjugated with aptamers for developing colorimetric and electrochemical based diagnosis methods discussed in the following sections.

Colorimetric Sensor

Colorimetric properties of nanoparticles are a function of their size and complexity. The most commonly used nanoparticles for colorimetric detection are gold and silver nanoparticles. Gold nanoparticles have a very high extinction coefficient. As discussed earlier, dispersed gold nanoparticles show reddish color while aggregated show blue color due to surface plasmon resonance shift to a higher wavelength (Mirkin et al. 1996). Chang et al. (2013) demonstrated the detection of platelet-derived growth factor (PDGF) using gold nanoparticles-aptamer bioconjugate by colorimetric detection (Fig. 9.4). The detection was based on base stacking effect coupled with unmodified gold nanoparticles as indicator. In the presence of target protein which binds to aptamer probe, followed by base stacking effect that resulted in a favourable and stable interaction between the aptamer and the capture probes.

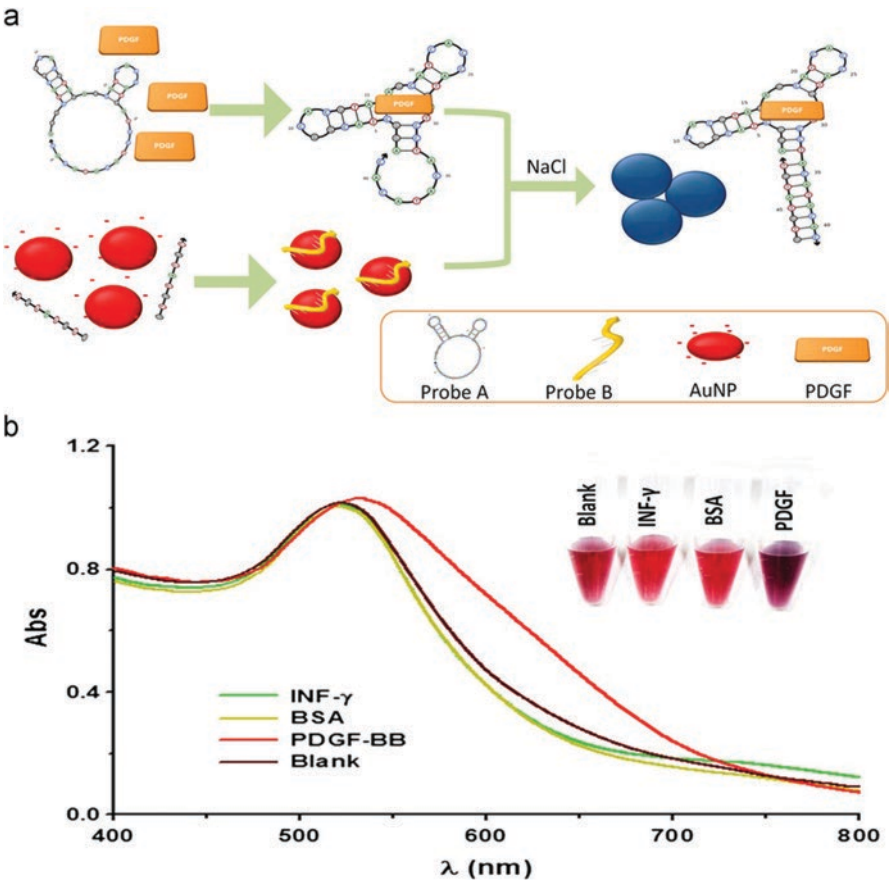


Fig. 9.4 (**a**) Schematic of the PDGF-BB detection design. (**b**) Absorption spectra for the aptasensor in the presence of specific and nonspecific proteins in 1X phosphate buffer saline. The concentrations of all proteins were 50 nM. Inset: Photographic images of the corresponding solutions. [Adapted from Chang, Wei (Chang et al. 2013) with permission from Elsevier]. (*PDGF* platelet-derived growth factor, *BB* aptamer probe, *IFN-γ* interferon γ, *BSA* bovine serum albumin, *NaCl* sodium chloride, *Abs* absorbance)

Further, capture probes dissociation from the gold nanoparticles surface induced their aggregation, while in the absence of PDGF, both the aptamer and the capture probe coexisted in solution because the complementary sequences were short (8 bp only). This label-free sensitive colorimetric sensor can detect up to 6 nM (Chang et al. 2013). Colorimetric sensing being more sensitive, many research groups focused on the development of nanoparticle-aptamer conjugate for the detection of biomarkers for a disease or disease-causing organism or even whole cell of infection agent. More recently, our group has developed a "turn-on" aptasensor based on the peroxidase-like activity of gold nanoparticles (regarded as NanoZyme) for the detection of small molecules like kanamycin and acetamiprid, a neurotoxic pesticide

(Weerathunge et al. 2014; Sharma et al. 2014). This approach gives a highly specific colorimetric output owing to the specific interaction of aptamer to its cognate target. This strategy yields highly sensitive detection down to 0.1 ppm acetamiprid and ~1.5 nM kanamycin (Sharma et al. 2014). Being a generic approach, this strategy may also be used for the detection of other complex targets like whole cells.

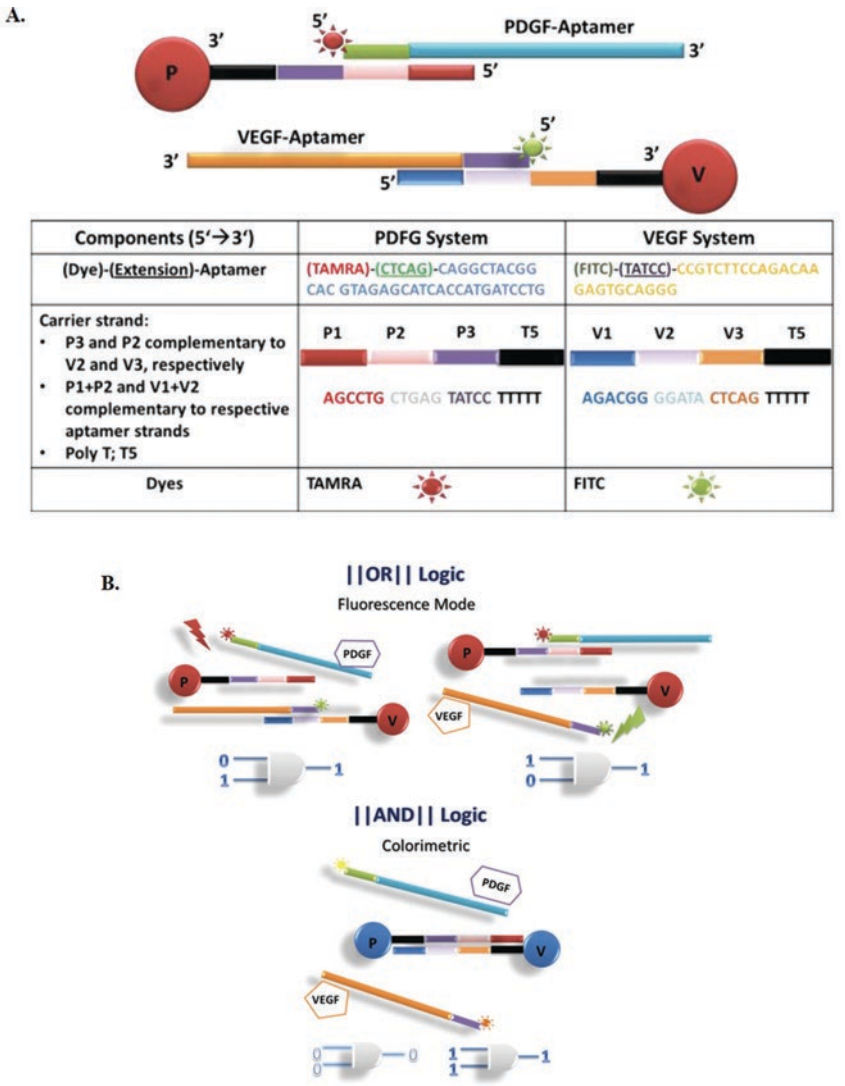
Optical sensors exhibit high sensitivity and specificity, but their sensitivity is reduced in presence of a colored sample which interferes with the detection mechanism. The diagnostic tool often employed for detection of analytes in colored samples such as blood is based on electrochemical sensing discussed as follows.

Electrochemical Sensor

For colored samples, electrochemical sensors are indispensable. Blood sample being colored can be easily monitored using electrochemical sensors. Lai et al. (2007) reported the detection of PDGF using an electrochemical, aptamer-based sensor directly in unmodified blood serum. They claimed that the sensor being very sensitive and highly selective could "detect the BB variant of PDGF at 1 nM directly in undiluted, unmodified blood serum and at 50 pM (1.25 ng/mL) in serum-diluted 2-fold with aqueous buffer" (Lai et al. 2007). Compared to optical detection methods, this approach has improved the detection limit by four orders of magnitude. Further, the aptamer-based sensing method is reusable, label-free, and electronic. These features encourage the implementation of electrochemical sensors in portable microdevices to be used on-site use for detection of protein and small molecules in clinical samples.

In a very interesting work, Shukoor et al. (2012) developed Boolean logic operations for the detection of PDGF and vascular endothelial growth factor (VEGF) (Fig. 9.5). "In this work, gold nanoparticles perform Boolean logic operations in response to two proangiogenic targets important in cancer diagnosis and treatment: PDGF and VEGF. In the absence of protein target, gold nanoparticles are initially dispersed as a red solution; the addition of target proteins causes nanoparticle aggregation, turning the solution blue, as well as the release of dye-labeled aptamer probes, which causes an increase in fluorescence. These outputs constitute an AND or OR gate for simultaneous protein detection. We believe this logic-gate-based detection system will become the basis for novel rapid, cheap, and reliable sensors for diagnostic applications" (quoted with permission from ACS). The developed sensor could spectrophotometrically detect protein concentrations as low as 1 nM; however, a clear visible color change from red to purple was observed at a protein concentration of 25 nM (Shukoor et al. 2012). The logic-based detection method due to its high specificity and sensitivity presents itself as a promising diagnostic tool.

Thus, the conjugation of nanostructures with aptamers has been utilized for development of sensors with fluorescence, optical, and electrochemical detection signals. Aptamer-nanoparticles-based sensing method is sensitive, highly specific, reusable, and label-free. It presents itself as a promising diagnostic tool for the



Components (5′→3′)	PDFG System	VEGF System
(Dye)-(Extension)-Aptamer	(TAMRA)-(CTCAG)-CAGGCTACGG CAC GTAGAGCATCACCATGATCCTG	(FITC)-(TATCC)-CCGTCTTCCAGACAA GAGTGCAGGG
Carrier strand: • P3 and P2 complementary to V2 and V3, respectively • P1+P2 and V1+V2 complementary to respective aptamer strands • Poly T; T5	P1 P2 P3 T5 AGCCTG CTGAG TATCC TTTTT	V1 V2 V3 T5 AGACGG GGATA CTCAG TTTTT
Dyes	TAMRA	FITC

Fig. 9.5 (**a**) Scheme 1. (Top) Two Types of Nanoparticles That Make up the Nanoparticle Logic Gate with No Target in the System and Each Aptamer (Purple and Orange) Protecting Its Complementary DNA. (Bottom) Components Used in the System. (**b**) Scheme 2. Schematic of Gold Nanoparticle-Based OR (Top) and AND (Bottom) Logic Gate Designs for Fluorescence Detection and Colorimetric Output Mode, Respectively. [Adapted from Shukoor, Altman (Shukoor et al. 2012) with permission from American Chemical Society (ACS)]

future. Apart from being wonderful sensors, nanoparticles have been successfully used to assist diagnostic imaging done by sophisticated techniques including magnetic resonance imaging, computed tomography, ultrasound, optical imaging, and photoacoustic imaging, as well as positron emission tomography and single photon emission computed tomography as pointed out in the subsequent section.

9.2.6 Nanostructures in Imaging

The unique properties of materials at the nanoscale make them suitable candidates for being explored to assist noninvasive imaging techniques such as magnetic resonance imaging, computed tomography, ultrasound, optical imaging, and photoacoustic imaging, as well as positron emission tomography and single photon emission computed tomography. The requisite properties of nanoparticles to make them suitable as contrast enhancement agents include high site specificity and appropriate blood circulation half-lives. While particles <5 nm have a very short circulation half-life due to uptake and clearance by the reticuloendothelial system, those >1 μm might have a very large circulation half-life due to no clearance or uptake by reticuloendothelial system increasing the background signal (Kiessling et al. 2014). Among the wide variety of nanomaterials available, iron oxide nanoparticles have been used as contrast agents in magnetic resonance imaging to either monitor gene expression or to detect cancer, inflammation, arthritis, or atherosclerotic plaques (Moghimi et al. 2005). Also, quantum dots have been employed for antigen, receptor, and enzyme imaging along with tracking of metastatic tumor cell extravasation (Akerman et al. 2002; Voura et al. 2004; Dahan et al. 2003; Wu et al. 2002).

Nanoparticle-aptamer conjugates have also been used in imaging methods such as magnetic resonance imaging and surface-enhanced Raman scattering (SERS). SERS imaging is based on "Raman scattering" which can be defined as the inelastic scattering of a photon by an excited molecule. Raman scattering can be amplified by employing SERS phenomenon. Wang et al. (2007) have shown the detection of α-thrombin by SERS. Two aptamers were selected for two binding sites of thrombin. Here in this work, they first immobilized aptamer 1 on a substrate and then treated with thrombin followed by gold nanoparticles functionalized with aptamer 2 and SERS reporter. The authors claimed the detection limit to be around 0.5 nM. Advantage of SERS is that it can be used for in vivo imaging also. Polyethylene glycol-modified nanoparticle-aptamer bioconjugate can be used for targeted imaging of tumors (Qian et al. 2007; Keren et al. 2008). Table 9.1 summarizes the use of different nanostructures for a wide variety of noninvasive imaging techniques used for diagnosing different pathophysiologies.

Therefore, nanostructures have improved medical diagnosis by providing inexpensive, reproducible, sensitive, and highly specific methods for disease diagnosis either in terms of sensors or as imaging agents. While a large variety of products have already reached market (Table 9.2), many are in different phases of clinical trials (Caster et al. 2017) or are being developed at laboratory scale to provide different tools and hence help understand the mechanisms in which normal and diseased cells differ, along with detecting disease-specific analytes. The magnificent properties of nanostructures have not only advanced nanodiagnostics, but the treatment of diseases has been explicitly enhanced by nanostructure-based delivery agents described in the rest part of the chapter.

Table 9.1 Different nanostructures employed for disease diagnosis using noninvasive imaging techniques (Baetke et al. 2015)

Imaging method	Nanoparticulate contrast agents developed
Magnetic resonance imaging	Gadolinium – containing probes
	(Ultrasmall) superparamagnetic iron oxide nanoparticles
	Paramagnetic liposomes and polymers
	ParaCEST agents
	Hyperpolarized probes
Computed tomography	Iodine-based micelles and liposomes
	Barium-based nanoparticles
	Gold-based nanoparticles
	Bismuth nanoparticles
Ultrasound	Targeted and nontargeted gas-filled microbubbles
	Nanobubbles
	Air-releasing polymers
Optimal imaging	Near-infrared fluorochrome-labeled nanoparticles
	Quantum dots
	Fluorescent nanoparticles probes
Photoacoustic imaging	Gold nanoparticles, gold nanorods
	Carbon nanotubes
	Fluorescent dye-loaded nanoparticles
Positron emission tomography	Radioactive contrast agents (e.g., radiolabeled gold nanoshells)
	Polymeric nanoparticles
Single photon emission computed tomography	Technetium-labeled gold nanoparticles
	Indium-labeled liposomes
	Nano- and microcolloids

Table 9.2 Different nanostructures in market for in vivo imaging and diagnostics (Wagner et al. 2006)

Market product or application	Nanostructure	Indication
In vivo imaging		
Resovist	Iron nanoparticles	Liver tumors
Feridex/Endorem	Iron nanoparticles	Liver tumors
Ferumoxsil	Siloxane-coated iron oxide nanoparticles	Oral contrast agent
Gastromark/Lumirem	Iron nanoparticles	Imaging of abdominal structures
Sienna	Dextran-coated iron oxide nanoparticles	Sentinel lymph node mapping
In vitro diagnostic		
Lateral flow tests (NicAlert, Verigene)	Colloidal gold	Pregnancy, ovulation, HIV, among others
Clinical cell separation (CellTracks)	Magnetic nanoparticles	Immunodiagnostics

9.3 Nanostructures for Drug and Gene Delivery

Nanostructures offer several advantages over conventional drug therapies, making them suitable candidates for drug and gene delivery applications. The small size of nanostructures, 5–100 nm, makes them suitable for staying in circulation for a longer time and increasing cellular uptake by various cells, thus increasing the probability of reaching the target site. Moreover, the size of nanostructures also makes them suitable candidates for enhanced permeability and retention effect which is helpful in treating cancer due to the leaky tumor vasculature (Kumar 2012; Greish 2010). The higher surface area to volume ratio of nanostructures increases drug dissolution, resulting in the drugs being effective at low dosage. Moreover, the bioavailability of drugs is increased by their association with nanostructures as they are protected against degradation while in circulation. Nanostructures also help in improving the pharmacokinetics of poorly water-soluble or insoluble drugs (Dreaden et al. 2012). Since conventional drug therapies involve systemic application of drugs, they do not just damage the diseased cells but even harm the healthy ones. The abovementioned issues can be circumvented by using different nanostructures such as micelles or liposomes and nanoparticles made of polymers, lipids, or inorganic material for drug or gene delivery. Targeted delivery of the therapeutic molecule by attaching antibodies and aptamers helps in specific and selective delivery, increasing the effectiveness of the drug. With targeted drug delivery, the concentration of the therapeutic molecule increases at the desired sites of action while other tissues remain unaffected. The following sections discuss applications of different nanostructures as antimicrobials for treatment of cancer, pain, asthma, multiple sclerosis, and kidney diseases.

9.3.1 Nanostructures for Cancer Treatment

Conventional therapies to treat cancer generally involve use of radiation and chemotherapy which cause significant damage to healthy cells as well. Nanostructures in the form of polymeric, lipid, inorganic, or magnetic nanoparticles are being extensively employed to treat cancer by active or passive targeting. The first liposomal formulation encapsulating anticancer drug Doxil was approved by Food and Drug Administration in 1995. Since then, a plethora of drugs including amphotericin B, daunorubicin, and morphine have been encapsulated in liposomes and are being marketed as commercial products (Table 9.3). Encapsulation of commonly used anticancer drug paclitaxel in albumin nanoparticles (130 nm) resulted in reducing the side effects (Micha et al. 2006) of the drug administered systematically along with higher drug dosing at tumor site (Ibrahim et al. 2002). Nanostructures have also been used for multiple drug therapy, for example, polymer poly-(lactic-co-glycolic) acid (PLGA) was used to encapsulate drug doxorubicin (chemotherapeutic) in liposomes composed of phospholipids conjugated with polyethylene glycol and

Table 9.3 Approved nanostructure products in market for drug and gene delivery applications (Wagner et al. 2006)

Disease category	Indication	Drug	Nanostructure	Market product
Infectious	Fungal infections	Amphotericin B	Lipid complex	Abelcet
			Lipid colloidal dispersion	Amphotec
			Liposome	Ambisome
	Hepatitis B and C	Interferon α-2a	Pegylated	Pegasys
	Hepatitis C	Interferon α-2b	Pegylated	PegIntron
Cancer	Breast cancer, pancreatic cancer, non-small cell lung cancer	Paclitaxel	Albumin-bound nanoparticle	Abraxane
	Kaposi's sarcoma	Daunorubicin	Liposome	DaunoXome
	Cancer, meningitis	Cytosine arabinoside	Liposome	Depocyte
	Kaposi's sarcoma, ovarian cancer, breast cancer, and multiple myeloma	Doxorubicin	Liposome	Doxil
	Breast, lung, and ovarian cancer	Paclitaxel	Polyethylene glycol-polylactic acid polymeric micelle	Genexol-PM
Cancer	Kaposi's sarcoma, breast and ovarian cancer	Doxorubicin	Liposome	Lipo-Dox
	Acute lymphoid leukemia	Vincristine	Liposome	Marqibo
	Osteosarcoma	Mifamurtide MTP-PE	Liposome	Mepact
	Breast cancer	Doxorubicin	Liposome	Myocet
	Thermal ablation glioblastoma	–	Iron oxide nanoparticle	NanoTherm
	Leukemia	L-Asparaginase	Polyethylene glycol protein conjugate	Oncaspar
	Acromegaly	–	Polyethylene glycol-human growth hormone	Somavert
	Liver and renal cancer	Styrene maleic anhydride neocarzinostatin	Polymer protein conjugate	Zinostatin stimalamer

(continued)

Table 9.3 (continued)

Disease category	Indication	Drug	Nanostructure	Market product
Neural	Multiple sclerosis	Glatiramer acetate	Copolymer of alanine, lysine, glutamic acid, and tyrosine	Copaxone
	Age-related macular degeneration	Anti-vascular endothelial growth factor	Pegylated aptamer	Macugen
	Age-related macular degeneration	Verteporfin	Liposome	Visudyne
Miscellaneous	Immunodeficiency disease	Adenosine deaminase	Pegylated drug	Adagen
	Antiemetic	–	Nanocrystalline aprepitant	Emend
	Menopausal therapy	Estradiol	Micellar nanoparticles	Estrasorb
	Severe combined immunodeficiency	Adenosine deaminase	Pegylated liposome	Exparel
	Postoperative pain	Bupivacaine	Liposome	Exparel
	Anemia of chronic kidney disease	–	Carbohydrate-coated iron oxide	Feraheme
	Eating disorder	Megestrol acetate	Nanocrystalline formulation	Megace ES
	Chemotherapy-induced neutropenia	Filgrastim	Pegylated-G-cerebrospinal fluid	Neulasta
	Immunosuppressant	–	Nanocrystalline sirolimus	Rapamune
	Chronic kidney disease	–	Cross-linked poly(allylamine) resin	Renagel
	Lipid regulation	–	Nanocrystalline fenofibrate	Tricor or Triglide

drug combretastatin (antiangiogenic) (Sengupta et al. 2005). The synthesized nanostructures, 80–100 nm in diameter, assist control release due to slow degradation of PLGA along with increased residence time due to polyethylene glycol conjugation (Harris and Chess 2003), thus increasing the effectiveness of drugs by enhanced permeation and retention effect. Recently, Hrkach and Von Hoff (2012) reported synthesis of polylactic acid-PLGA nanoparticles containing anticancer drug docetaxel, which were targeted to prostate-specific membrane antigen. The synthesized nanoparticles were tested for their efficacy in mouse tumor models, and their pharmacokinetics was tested in mice, rats, monkeys, and humans.

Cationic liposomes, solid lipid nanoparticles are also being used to deliver nucleic acid-based therapeutics including small interfering RNA, antisense oligonucleotides, and aptamers (Farokhzad and Langer 2006). Active targeting of different nanostructures often involves attaching receptor molecules such as antibodies and aptamers on their surface that bind with antigens/molecules expressed on cancer cells (Kumar 2012). Different nanostructures being marketed for use in cancer treatment are summarized in Table 9.3 and those approved for different clinical phase trials in Tables 9.4 and 9.5. While cancer is the major target of most of the nanostructures being developed, nanostructures are also being engineered for treatment of diseases caused by microbial infections.

9.3.2 Nanostructures as Antimicrobials

Nanostructures not only act as delivery vehicles for delivery of different antimicrobials, but polymeric and metallic nanoparticles have themselves demonstrated antimicrobial activity (Caster et al. 2017; Sharma et al. 2012a, b). The renal and neurological toxicity of antibiotic aminoglycoside, a drug useful for treating multidrug-resistant tuberculosis and gram-negative bacteria, was prevented by encapsulating the drug in a liposomal formulation, which also increased the circulation time of the drug (Caminero et al. 2010; Canton et al. 2005; Meers et al. 2008). Inhalable liposomal formulation for drug-resistant pseudomonas infections is under clinical trials to treat patients with cystic fibrosis. Amphotericin B, an antifungal agent, is also being marketed as a liposomal formulation (Larson et al. 2000). Various dendrimeric and polymeric formulations containing antibiotic, antibacterial, and antifungal drugs are being developed to prevent these infections. Different polymeric and metallic nanoparticles are themselves being used as antimicrobials. While quaternary ammonium polyethyleneimine, a highly charged polymer molecule, has been employed to disrupt the membrane of gram-positive and gram-negative bacteria (Ortega et al. 2015), nano silver has been used to induce toxic effects. Nano silver kills bacteria by release of ions that can easily penetrate the organism. In a recent study by Sharma and Sapra (Sharma et al. 2012b), silver nanoparticles were functionalized in a single step with an antibacterial peptide, enterocin, from a food-grade lactic acid bacterium. The synthesized nanoparticles exhibited broad-spectrum inhibition without any detectable toxicity to red blood cells. Inhibition in the growth of *Staphylococcus aureus* and *Escherichia coli* was determined using scanning electron microscope and has been depicted in Fig. 9.6 (Sharma et al. 2012b).

Protein-based nanoparticles containing respiratory syncytial virus fusion protein have been developed to treat respiratory syncytial virus. Different nanostructures being marketed for use in treatment of infectious diseases are summarized in Table 9.3 and those approved for different clinical phase trials in Tables 9.4 and 9.5.

Table 9.4 Approved nanostructure products in clinical trials for drug delivery applications (Caster et al. 2017)

Disease category	Indication/investigational use	Drug	Nanostructure	Product name	Clinical trial phase
Infectious	Gram-negative, pseudomonal, Mycobacterium-avium complex infections	Amikacin	Liposome	Arikace	III
	Gram-negative infections	Ciprofloxacin	Liposome	Lipoquin	II
				Pulmaquin	II–III
		Amikacin	Lipid/crystal	MAT2501	New drug application
	Fungal infections	Nystatin	Liposome	Nyotran	III
	Human immunodeficiency virus infection	Efavirenz	Polymeric	NANOefavirenz	I
		Lopinavir		NANOlopinavir	I
	Herpes simplex virus, human immunodeficiency virus infections	Astodrimer	Dendrimer	Vivagel	II–III
	Topical infections	Silver	Hydrogel	Silvasorb	III
	Blepharitis	Loteprednol etabonate	Liposome	KPI 121	II
	Pneumonia	CAL02	Liposome	*	I
	Mixed oral flora infections	Ammonium polyethyleneimine	Polymeric	QA-PEI	I–II
	Chronic candidiasis	Amphotericin B	Lipid/crystal	MAT2203	II
	Respiratory syncytial virus infections	RSV fusion protein	Protein	RSV-F	II
	Bronchiolitis obliterans	Cyclosporin	Liposome	L-CsA	I–II
	Interstitial cystitis	Botulinum toxin A	Liposome	Lipotoxin	I–II
	Cutaneous leishmaniasis	Amphotericin B	Liposome	*	II
	Chronic periodontitis	Doxycycline	Polymeric	*	II

(continued)

Table 9.4 (continued)

Disease category	Indication/investigational use	Drug	Nanostructure	Product name	Clinical trial phase
Cancer	Metastatic breast cancer	Doxorubicin	Liposome	MM-302	II
		Paclitaxel	Polymer micelle	NK-015	III
	Metastatic breast cancer, non-small cell lung cancer	Paclitaxel	Polymer micelle	Genexol-PM	III
	Metastatic cancer	Irinotecan	Liposome	MM-398	III
	Metastatic prostate cancer, non-small cell lung cancer	Docetaxel	PSMA-targeted polymeric micelle	BIND-014	II
	Advanced solid tumor malignancies	Irinotecan	Liposome	IHL-305	I
		Mitomycin C		Promitil	I
		SN-38 (irinotecan metabolite)	Polymer micelle	NK-012	I/II
		Cisplatin		Nanoplatin	II/III
		Oxaliplatin		NC-4016	I
	Advanced solid tumor malignancies, rectal cancer	Camptothecin	Cyclodextrin conjugate	CRLX-101	I/II
	Hepatobiliary tumors (with radiofrequency ablation)	Doxorubicin	Liposome	Thermodox	III
	High-risk acute myelogenous leukemia	Daunorubicin + cytarabine	Liposome	CPX-351	II
	Gynecological malignancies	Paclitaxel	Polymer micelle	Paclical	III
	Head and neck cancer, glioblastoma multiforme, gynecological malignancies	Paclitaxel	Poliglumex	Opaxio	II/III
	Refractory tumors	Docetaxel	Polymer conjugated	CRLX-301	I (planned)
	Advanced cancer	Docetaxel	Dendrimer conjugated	DTX-SPL8783	I
	Advanced gastric cancer	Doxorubicin	Polymer micelle	SP1049-C	III

Neural	Schizophrenia	Curcumin	Colloid	Theracurmin	I–II
		Glutathione	Liposome	ReadiSorb Glutathione	I
	Oculomotor apraxia type I	Coenzyme Q10	Liposome	*	III
	Ocular hypertension, glaucoma	Latanoprost	Liposome	POLAT-001	II
	Diabetic neuropathy	Capsaicin	Liposome	Capsaicin Cream	II–III
Miscellaneous	Coronary restenosis post percutaneous coronary intervention	Alendronate	Liposome	LABR-312	II
	Rheumatoid arthritis, ulcerative colitis	Prednisolone	Liposome	Nanocort	III
	Atopic dermatitis	Adenosylcobalamin	Liposome	HL009	II
	Radiation dermatitis	Recombinant Cu/Zn superoxide dismutase	Liposome	APN201	I–II
	Analgesia	Fentanyl	Liposome	AeroLEF	I
	Actinic keratosis	T4 endonuclease V protein	Liposome	T4N5	III
	Hemophilia A	Recombinant factor VIII	Liposome	BAY-79-4980	I
	Peripheral arterial disease	Prostaglandin E	Liposome	Lipostin	II
	Diabetes	Insulin	Micelle	HDV Insulin	II–III
	Menopausal symptoms	Estrogen/progesterone	Liposome	*	II
	Menopausal symptoms	Testosterone	Liposome	*	II
	Vitamin D deficiency	Vitamin D	Micelle	*	I
	Gout	Uricase	Polymeric	SEL-212	I
		Rapamycin	Polymeric	SEL-110	I
	Diabetic macular edema	Dexamethasone	Polymeric	*	II–III
	Hepatic fibrosis	Vitamin A	Liposome	ND-L02-s0201	I
	Renal dialysis	Prednisolone	Liposome	LIPMAT	II
	Femoral fracture	Bupivacaine	Liposome	*	II
	Acne	Povidone iodine	Liposome	Repigel	II

*No product name listed

Table 9.5 Approved nanostructure products in clinical trials for gene delivery applications (Caster et al. 2017)

Disease category	Indication	Target	Drug	Clinical trial phase
Infectious	Ebola virus infection	VP24, VP35, Zaire ebola, L-polymerase	TKM-100201	I
	Respiratory syncytial virus infection	Respiratory syncytial virus nucleocapsid	ALN-RSV01	II
	Hepatitis B virus infection	HBV conserved domains	ARC-520	I
Cancer	Adult, pediatric tumors	p53	SGT-53	II
	Pancreatic cancer	KRAS	siG12D-LODER	II
	Solid tumor malignancies	KSP and VEGF	ALN-VSP	I (extension trial recently completed)
	Solid tumor malignancies	RRM2	CALAA-01	I (terminated)
	Solid tumor malignancies	cRaf	LErafAON	I
	Solid tumor malignancies	RX34 microRNA	miR-RX34	I
	Solid tumors	PKN3	Atu027	I
	Recurrent solid tumors	EphAS	EphA2-DOPC	I
	Non-small cell lung cancer	Fus 1 gene (insertion)	DOTAP-Chol-EGFR	I
	Oral squamous cell carcinoma	EGFR	DC-Chol-EGFR	I
	Cancer	PLK1	TKM-080301	I
	Advanced cancers	EphA2	Small interfering RNA-EphA2-DOPC	I
Neural	Pachyonychia congenita	K6a (N1 71 K mutation)	TD101	I
	Age-related macular degeneration, choroidal neovascularization	VEGFR1	AGN211745	II
	Ocular pain, dry eye syndrome	TRPV1	SYL1001	I/II
	Ocular hypertension, open-angle glaucoma	ADRB2	SYL040012	II
	Optic atrophy, nonarteritic anterior ischemic optic neuropathy	CASP2	QPI-1007	I

(continued)

Table 9.5 (continued)

Disease category	Indication	Target	Drug	Clinical trial phase
Miscellaneous	Kidney injury, acute renal failure	p53	I5NP	I/II
	Choroidal neovascularization, diabetic retinopathy, diabetic mascular edema	RTP801 (proprietary target)	PF-655 (PF-04523655)	II
	Diabetic mascular edema, mascular degeneration	VEGF	Bevasiranib	II
	Familial adenomatous polyposis	CTNNB1	CEQ508	I/II
	Cicatrix scar prevention	CTGF	RXi-109	I
	Transthyretin-mediated amyloidosis	Transthyretin	Patisiran (ALN-TTR02)	II/III
			ALN-TTRsc	I
			ALN-TTR02	II
	Hypercholesterolemia	ApoB	PRO040201	I (terminated)
		PCSK9	ALN-PCS	I (completed)

KRAS Kirstin Rat Sarcoma Viral Oncogene Homologue, *KSP* Kinesin Spindle Protein, *VEGF* Vascular Endothelial Growth Factor, *PCSK9* Proprotein Convertase Subtilisin/Kexin Type 9, *RRM2* Ribonucleotide Reductase 2, *ApoB* Apolipoprotein

9.3.3 Nanostructures for Other Diseases

Nanostructures are also being explored for increasing the efficacy of drugs associated with ophthalmic conditions, pain management, psychological illness, metabolic disorders, as well as neurological, cardiovascular, respiratory, and autoimmune diseases. These have been discussed briefly in the subsequent paragraphs.

Blood-brain barrier is one of the major turning stones in treating diseases associated with central nervous system. Blood-brain barrier protects the brain cells by not allowing foreign substances to enter and thus prevents therapeutic molecules to enter. As a consequence, the amount of drug administered is very high, resulting in adverse side effects. However, nanoparticles have demonstrated the ability to cross the blood-brain barrier and effectively deliver the drugs to brain cells. Doxorubicin is unable to cross the blood-brain barrier when given directly; however, its association with polysorbate 80-modified polybutylcyanoacrylate nanoparticles facilitates its delivery to the brain (Gulyaev et al. 1999). An analgesic drug dalargin was delivered to the central nervous system by adsorbing onto poly(butyl cyanoacrylate) (PBCA) nanoparticles (Kreuter et al. 2003). PBCA has also been used to treat Alzheimer's disease (Siegemund et al. 2006). Other polymers that have been employed include poly(hexadecyl cyanoacrylate) with polyethylene glycol,

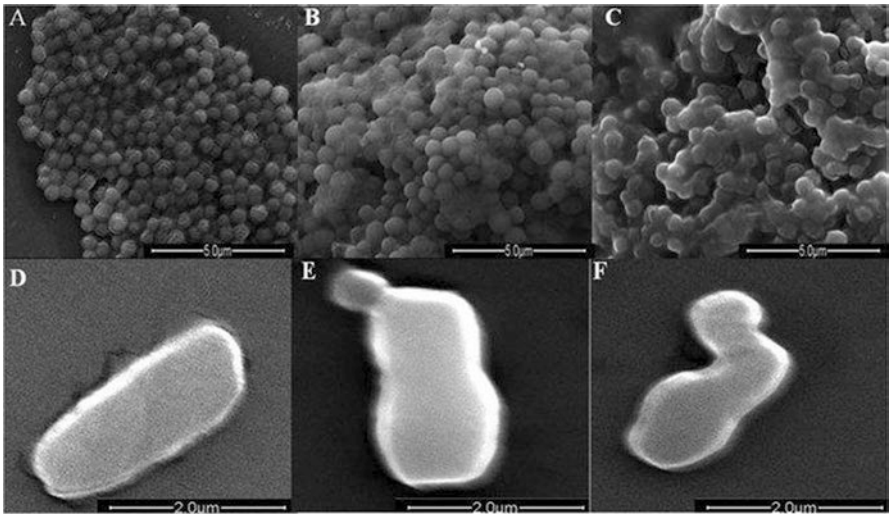


Fig. 9.6 Interaction of enterocin-capped silver nanoparticles with bacteria as observed through scanning electron microscope. Upper panel, *Staphylococcus aureus* (**a**) untreated, (**b**) treated with citrate-capped silver nanoparticles, and (**c**) treated with enterocin-capped silver nanoparticles. Lower panel, *Escherichia coli* (**d**) untreated, (**e**) treated with citrate-capped silver nanoparticles, and (**f**) treated with enterocin-capped silver nanoparticles (Sharma et al. 2012b)

poly(propylene oxide), and P-85 polymer. Polyethylene glycol-conjugated liposomes have also demonstrated their potential to deliver drugs across the blood-brain barrier. Prednisolone, a drug used to treat multiple sclerosis, was delivered using polyethylene glycol-conjugated liposomes 90–100 nm in diameter (Schmidt et al. 2003). Nucleic acid-based therapeutics have also been developed using antibodies conjugated to liposomes which have demonstrated their potential in rat models (Shi et al. 2001).

Asthma, a very common allergic respiratory disease, is caused by drop in production of interferon-γ (IFN-γ), which results in the patient being susceptible to inflammation of the airways. Kumar et al. (Kumar et al. 2003) developed a polymer-drug conjugate comprising of chitosan and IFN-γ pDNA which was delivered intranasally to increase the production of IFN-γ and was reported to reduce inflammation. Liposome-based 73 nm nanostructures have also been developed for treatment of allergic asthma (John et al. 2003). However, the development of these nanostructures is still in inception and would need clinical trials to be brought to human use.

Nanostructures have also found applications in treating human immunodeficiency virus (HIV), acquired immunodeficiency syndrome, and different ophthalmic conditions. HIV-1 protease inhibitor delivery is being investigated by Allemann et al. using commercially available polymer Eudragit (De Jaeghere et al. 2000). Solid lipid nanoparticles prepared using cationic lipids and surfactants have been employed to adsorb HIV-1 Tat protein and DNA on the surface of nanoparticles (Rudolph et al. 2004). Treatment of ophthalmic conditions is often associated with

the ability of the drug to get entrapped in the ocular mucus layer that protects the epithelial layer of cornea for a long duration. Nonsteroidal anti-inflammatory drugs flurbiprofen and ibuprofen have been delivered to rabbit eyes employing nanoparticles ~ 100 nm in size using polymers such as poly(ethyl acrylate), poly(methyl methacrylate), poly(chlorotrimethyl-aminoethyl-methacrylate), and commercially available Eudragit (Pignatello et al. 2002a, b). These polymers assist controlled release due to their properties of being insoluble and capability of swelling under physiological conditions.

Nanostructures including solid dispersions, liposomes, nanoemulsions, self-emulsifying drug delivery systems, nanostructured lipid carriers, cyclodextrins, and nanocapsules have been employed for delivery of coenzyme Q10. Coenzyme Q10 is an antioxidant and essential molecule required for growth of every cell in the body. Deficiency of Coenzyme Q results in disorders including neurological degeneration, reproductive disorders, aging, and cancer. The use of nanostructures for delivery of coenzyme Q10 has resulted in reduced oxidative stress, increased bioavailability, controlled release, and increased dissolution rates (Paroha et al. 2018).

In comparison to cancer and infectious diseases, not many products have reached the nanomedicine market for treatment of ophthalmic conditions, pain management, psychological illness, metabolic disorders, as well as neurological, cardiovascular, respiratory, and autoimmune diseases. Table 9.3 summarizes the approved products available in market for treatment of the mentioned diseases. However, many nanostructures are under clinical trials (Tables 9.4 and 9.5) and in developmental stages to be used as effective drug and gene delivery vehicles. Nanostructures have also been conjugated to aptamers in order to enhance delivery efficacy. Aptamer-nanostructure conjugates discussed below, due to their high binding affinity and specificity, low immunogenicity, and versatile synthetic accessibility, offer themselves as promising therapeutic agents.

9.3.4 Aptamer-Nanoparticle Conjugates for Therapeutic Purposes

Nanoparticle-aptamer biconjugates along with varied diagnostic applications are used for therapeutic purposes as well (Ghosh et al. 2013; Lambadi et al. 2015). Aptamers with ease of selection and synthesis; high binding affinity and specificity; low immunogenicity; and versatile synthetic accessibility are good candidates for drug delivery in vivo. Different chemotherapy drugs like doxorubicin, docetaxel, daunorubicin, cisplatin, toxins such as gelonin along with various photodynamic therapy agents and small interfering RNAs can be delivered in the host body in a programmed manner using nanoparticle-aptamer bioconjugates.

In one report, the authors used nanoparticle-aptamer conjugate which encapsulated docetaxel. It was found that after endocytosis, the nanoparticle-aptamer-docetaxel conjugate bound to prostate-specific membrane antigen on the cancer cell

surface caused cellular toxicity while the control nanoparticle-docetaxel did not cause toxicity to the cancer cell (Farokhzad et al. 2006; Bleickardt et al. 2002; Miller and Kris 2000). Similarly, cisplatin, an anticancer drug, was delivered into cancer cells (Dhar et al. 2008). In another work by Bagalkot et al. (Bagalkot et al. 2007), smart quantum dot-aptamer conjugate was designed which served both as a fluorescence imager and a drug delivery vehicle. Different components were quantum dots, prostate cancer cell-specific RNA aptamer, and doxorubicin. In the presence of cancer cells, quantum dot-aptamer (doxorubicin) system gradually released doxorubicin induced by the binding of target molecule onto RNA aptamer. This Dox release recovered the fluorescence of the quantum dot. So, the quantum dot-aptamer (doxorubicin) system allows both targeted drug delivery and imaging target cells (Bagalkot et al. 2007).

9.4 Further Applications of Nanostructures as Nanomedicine

Nanomedicine not only includes the fields of therapeutics and diagnostics but also involves development of implantable materials and devices. Implantable materials basically include materials for tissue repair/regeneration, bone implants, and implant coatings. Surgical aids, implantable sensors, and sensory aids fall under the category of implantable devices (Agrawal 2016; Lambadi et al. 2015).

Success of an artificial bone implant lies on its integration with the human body which involves generation of osteoblasts on the implant, resulting in minimum chances of rejection. If implant surfaces are left smooth, the surfaces get covered by fibrous tissue resulting in loosening of implant and inflammation. Thus, covering the implant surfaces with nanostructures made of polymers (nano-hydroxyapatite), ceramics, and metals results in efficient integration of osteoblasts to the implant. Coating of nanostructures has given rise to development of long-lasting and durable implants (Sato et al. 2008). In order to improve scratch resistance of human teeth, an artificial hybrid of poly(methyl methacrylate) copolymer and 15–18 nm ceramic nanoparticles is available in market (de la Isla et al. 2003). Also, spherical, functionalized magnetic nanoparticles have been used for cell separation and probing (Pankhurst et al. 2003). Table 9.6 summarizes different nanostructure products available in market as bone substitutes, dental composites, device coatings, medical dressings, dialysis filter, and tissue scaffolds. A wide variety are still under development and would benefit the nanomedicine market in near future.

Despite the innumerable advantages of nanostructures in the field of nanomedicine, only a handful of products have been able to reach the market due to several disadvantages that these magic bullets are associated with. However, maintenance of a balance between the advantages and disadvantages would definitely open up avenues for personalized medicine through therapeutics, diagnostics, and theranostics.

Table 9.6 Nanostructure products available in market for various applications

Application	Product	Nanostructure
Bone substitute	Vitoss	100-nm calcium phosphate nanocrystals
	Ostim	2-nm hydroxyapatite nanocrystals
	OsSatura	Hydroxyapatite nanocrystals
	NanOss	Hydroxyapatite nanocrystals
	Perossal	Hydroxyapatite nanocrystals
	α-bsm, β-bsm, γ-bsm,	Hydroxyapatite nanocrystals
	EquivaBone, CarriGen	Ceramics nanoparticles
Dental repair	Ceram X Duo	Silica and zirconium nanoparticles
	Filtek Supreme	Nanoparticles containing dental
	Mondial	Prosthesis
	Nano-Bond	Nanoparticle composite
	Premise	Nanoparticle composite
	Tetric EvoCeram	Nanoparticle composite
Device coating	ON-Q SilverSoaker/SilvaGard	Antimicrobial nano silver
	EnSeal Laparoscopic Vessel Fusion	Nanoparticle-coated electrode
	NanoTite Implant	Calcium phosphate nanocrystal coating
Medical dressing	Acticoat	Antimicrobial nano silver
Dialysis filter	Fresenius Polysulfone Helixone	Nanoporous membrane
Tissue scaffold	TiMESH (lightweight polypropylene mesh)	30-nm titanium coating
Heart failure	Pacemaker	Fractal electrodes

9.5 Darker Side of Nanomedicine and Future Perspective

Applications of nanostructures in the field of diagnostics, therapeutics, and development of implantable materials have helped the medicine industry because of several advantages that these nanostructures offer. Nanomedicine has resulted in higher specificity, increased sensitivity, and faster response time at a much lower price as compared to conventional drug therapies. Nanobiosensors help in detection of very low concentrations of analyte under consideration and have also assisted targeted delivery (Sharma et al. 2015). Nanomedicine has helped treat a wide range of illnesses including cancer, pain, asthma, multiple sclerosis, and kidney diseases (Manoharan et al. 2018). While nanomedicine is becoming an important part of modern medicine, toxicity of the developed nanostructures to humans and the environment is a major concern. Disposal of nanostructures used for diagnosis of infectious diseases is a serious environmental concern. Nanostructures developed for therapeutic purposes are in very preliminary stages, and the long-term effects on human health require further investigation. While toxicity is a major concern, studies are being done to reduce the toxicity of the synthesized nanostructures weighing the benefits provided by them. For example, toxic CdSe quantum dots have been coated with ZnS/polyethylene glycol to reduce their toxicity (Ballou et al. 2004).

Naturally available polymers and lipids that are biocompatible and biodegradable are being employed to address the toxicity issues. Coating of nanostructures with different natural polymers such polyethylene glycol or proteins has been demonstrated to reduce toxicity to great extent (Goodman et al. 2004; Abraham et al. 2018). However, with risks associated with production, handling, and storage of these nanostructures in terms of loss of efficiency, toxicity needs to be understood further. Nanostructures possess the ability to integrate diagnostic and therapeutic applications in a single nanoparticulate formulation, making them promising candidates for theranostic applications which would help in personalizing nanomedicine-based treatment. Thus, in summary, nanostructures have shown great potential to play an important role in future development of diagnostics, therapeutics, and theranostic applications contributing to personalized medicine—the need of hour of modern medicine.

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Chapter 10
Biomedical Applications of Iron- and Cobalt-Based Biomagnetic Alloy Nanoparticles

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Abstract Nanotechnology has allowed scientists, materials engineers, chemists and physicians to work at the molecular and cellular levels due to its important advances in the life sciences and healthcare. The use of nanoparticle materials offers major advantages due to their unique size and physicochemical properties. Magnetic alloy nanoparticles are attractive materials due to widespread applications in various fields such as biotechnology, medical, material science and engineering. In this regard attention has been paid to the synthesis of various biomagnetic alloy nanoparticles (BMANPs). The biocompatibility and physical properties of these materials provide a very promising future for their use in biomedicine. Preparation of nanoparticles consisting of pure iron is a complicated task, because they usually contain oxide compounds, carbides and other impurities. Synthesis of pure iron nanoparticles is a complicated process because they usually contain oxide/carbides compounds. Besides, very high reactivity, toxicity and intrinsic instability of some nanoparticles caused to focus on coating nanoparticles by biocompatible materials. For example, the toxicity of cobalt nanoparticles is due to the cobalt leakage, which can be improved by inorganic encapsulation of cobalt, for example, with silica, hydroxyapatite, chitosan and sort of that. In addition, high sensitivity to oxidation of magnetic nanoparticles can be solved partially by coating or alloying such as gold, platinum, cobalt, carbon, etc. Hence, the synthesis and characterization of iron- and cobalt-based magnetic nanoparticles with biomedical applications is the purpose of this review.

We have reviewed different synthetic procedures which can partially solve the existing issue on magnetic nanoparticles such as micro-emulsion and polyol methods. The various surface modification technologies used to reduce the oxidation rate and toxicity are also included. The control of parameters to optimize the physical-chemical properties of nanoparticles is a key focus of this review. Two general fields of applications, namely, diagnosis (analytical biosensor/nucleotide interactions or visual bioimaging) and transportation (drug delivery and gene transfection), are discussed.

Keywords Iron · Cobalt · Magnetic · Nanoparticles · Biomedical · Applications

10.1 Introduction

10.1.1 Nanomedicine: Nanotechnology in Medicine

Recent years have witnessed an unprecedented growth in research in the area of nanoscience. One of the most promising applications of nanoscience is in the field of medicine. There is increasing optimism that nanotechnology applied to medicine will bring significant advances in the diagnosis treatment and prevention of diseases; however, many challenges must be overcome if the application of nanomedicine is to realize an improvement in the understanding of the pathophysiological basis of diseases. Nanomedicine embraces five main subdisciplines which in many ways are in overlap. These are underpinned by analytical tools, nano-imaging, nanomaterials and nano-devices, novel therapeutics and drug delivery systems and clinical, regulatory and toxicological issues. Miniaturizations of devices, chip-based technologies and sophisticated novel nano-sized materials and chemical assemblies are already providing novel tools that contribute to improved healthcare in the twenty-first century. Opportunities include superior diagnostics and biosensors, improved imaging techniques from molecules to man and, not least, innovative therapeutics and technologies to enable tissue regeneration and repair. However, to realize nanomedicine's full potential, important challenges must be addressed.

New regulatory authority guidelines must be developed quickly to ensure safe and reliable transfer of new advances in nanomedicine from the laboratory to bedside. These aspects were viewed as complementary even though many of the technologies required are very different in being designed for an ex vivo, cellular or in vivo/patient use (Nochehdehi et al. 2017a). Advances should begin with the optimization of existing technologies towards specific nanomedicine challenges. The development of new multifunctional, spatially ordered, architecturally varied systems for targeted drug delivery was seen as a priority. There is a pressing need to enhance expertise in scale-up manufacture and material characterization and to ensure material reproducibility, effective quality control and cost-effectiveness. These issues should be addressed urgently to enable rapid realization of clinical benefits within 5 years. For realization to application within the next decade, new materials are needed for sensing multiple, complicated analyses in vitro, for applications in tissue engineering, regenerative medicine and 3D display of multiple biomolecular signals.

Telemetrically controlled, functional, mobile in vivo sensors and devices are required, including construction of multifunctional, spatially ordered, architecturally varied systems for diagnosis and combined drug delivery (theranostics). The advancement of bioanalytical methods for single-molecule analysis is seen as a priority. Nano-sized drug delivery systems have already entered routine clinical use in this field. The most pressing challenge is the application of nanotechnology to the

design of multifunctional, structured materials which are able to target specific diseases or containing functionalities to allow transport across biological barriers. In addition, nanostructured scaffolds are urgently needed for tissue engineering, stimuli-sensitive devices for drug delivery and tissue engineering and physically targeted treatments for local administration of therapeutics, e.g. via the lung, eye or skin. To realize the desired clinical benefits rapidly, the importance of focusing the design of technologies on specific target diseases was stressed: cancer and neurodegenerative and cardiovascular diseases were identified as the priority areas.

Longer-term priorities include the design of synthetic, bio-responsive systems for intracellular delivery of macromolecular therapeutics, synthetic vectors for gene therapy and bio-responsive or self-regulated delivery systems including smart nanostructures such as biosensors that are coupled to the therapeutic delivery systems (Kostiv et al. 2017). There is an urgent need to improve the understanding of toxicological implications of nanomedicines in relation to the specific nanoscale properties currently being studied, in particular in relation to their proposed clinical use by susceptible patients. The nanoscale is the place where the properties of most common things are determined just above the scale of an atom. Nanoscale objects have at least one dimension (height, length, depth) that measures between 1 and 999 nm. According to Fig. 10.1, there are many objects in nanoscales that can be compared by the size of the spectrum (Afghahi and Shokuhfar 2014).

In addition, due consideration should be given to the potential environmental impact; there should be a safety assessment of all manufacturing processes. Risk-benefit assessment is needed in respect of both acute and chronic effects of nanomedicines in potentially predisposed patients – especially in relation to target disease. A shift from risk assessment to proactive risk management is considered essential at the earliest stage of the discovery and then the development of new nanomedicines (Nochehdehi et al. 2015). As the technologies are designed based on a clear understanding of a particular disease, disease-specific oriented focus is required for the development of novel pharmaceuticals. In addition, it will be important to establish a case-by-case approach to clinical and regulatory evaluation of each nano-pharmaceutical. High priority should be given to enhancing communication and exchange of information among academia, industry and regulatory agencies encompassing all facets of this multidisciplinary approach (Vencken and Greene 2018).

10.1.2 Magnetic Properties

The study of magnetic properties of materials at the nanoscale is an important area for the advancement of nanoscience and nanotechnology. It can be attributed to the fact that the nanoscale magnetic properties differ from their bulk counterparts. Therefore, magnetic nanoparticles in the size range of 1–100 nm have attracted a great deal of attention due to their technological importance. The research has

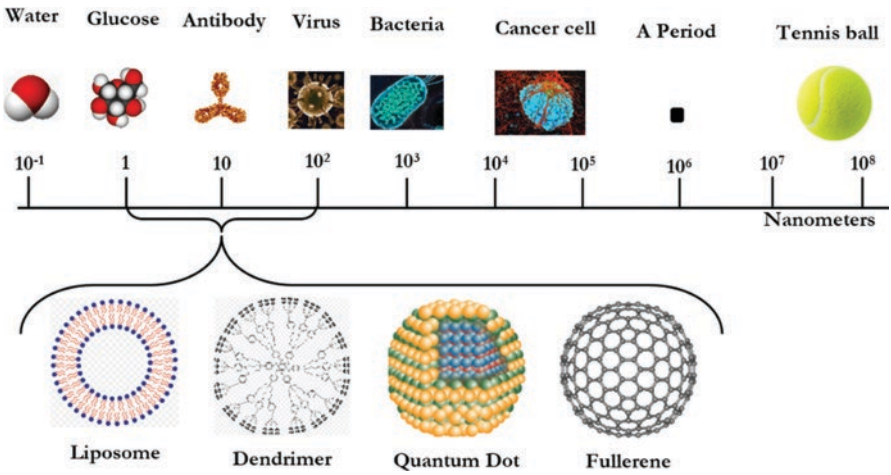

Fig. 10.1 The schematic size of spectrum and some nano-objects, in the range of 1–100 nm liposome, dendrimer and quantum dots can be synthesized in the same range as glucose or antibodies (Afghahi and Shokuhfar 2014)

evolved to develop nanoparticles in applications such as magnetic resonance imaging (MRI) for medical diagnosis (MD), high-density magnetic recording (HDMR), magneto-optical switches (MOS) and controlled drug delivery (CDD). Figure 10.2 summarized the above-described behaviour of materials when a magnetic field is applied (Kudera et al. 2016; Zverev et al. 2018).

Basically, all materials can be divided into three categories according to their interaction with an external magnetic field: diamagnetism, paramagnetism and cooperative magnetism (Kudera et al. 2016; Nicolas-Boluda et al. 2018; Rhodes 2018; Tekade et al. 2017; Zverev et al. 2018). The electron possesses a spin that is equivalent to the strength of the magnetic field (magnetic moment) of the electron itself, in an atom. Electrons are arranged in energy states of successive order; for each energy state, there can only be two electrons of opposite spins, as established by Paul Lange's principle. The orbital motion of an unpaired electron around the nucleus and the spin of the electron about its own axis can generate magnetic moments. The magnetic moment of each electron pair in an energy level is opposed, and consequently, whenever an energy level is completely full, there is no net magnetic moment. Based on this reasoning, we expect any atom of an element with an odd atomic number to have a net magnetic moment from the unpaired electron. In most of the elements, the unpaired electron is in the valence shell and can interact with other valence electrons leading to the cancellation of the net magnetic moment in the material. However, certain elements such as cobalt and nickel have an inner energy level that is not completely filled, i.e. each atom in the metal has a permanent magnetic moment, equal in strength to the number of unpaired electrons (Kudera et al. 2016).

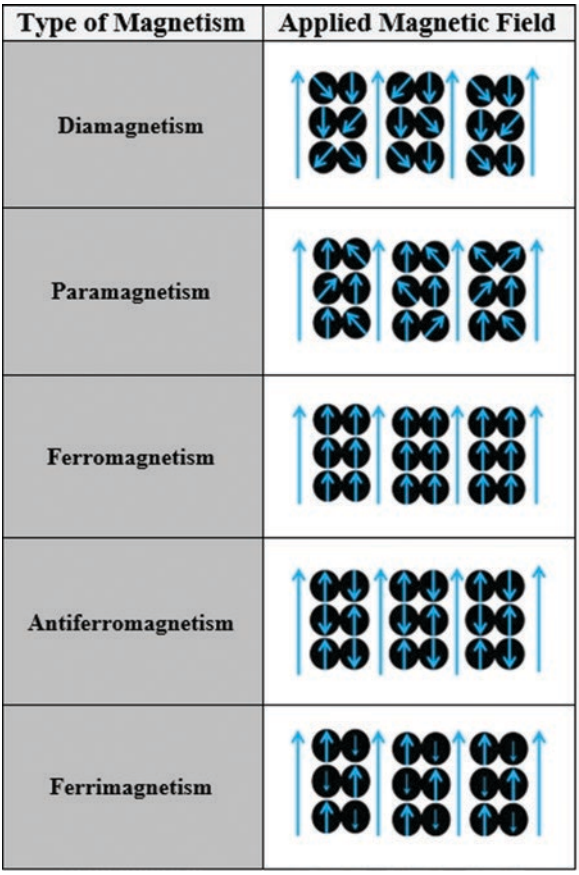

Fig. 10.2 The types of magnetism seen in materials. Blue arrows signify the direction of the applied field. Blue arrows in the black circle signify the direction of the electron spin (Kudera et al. 2016)

Diamagnetism

Diamagnetism refers to a material that exhibits a negative magnetism. Even though the material is composed of atoms that have no net magnetic moment (paired electrons), it reacts in a particular way to an applied field. Wilhelm Weber and Paul Lange theorized that an applied field acts on a single electron orbit to reduce the effective current of the orbit, in turn producing a magnetic moment that opposes an applied field. Common diamagnetic materials are water, wood, most organic compounds and in the case of this work copper (Kudera et al. 2016; Zhang 2018).

Paramagnetism

In other cases, atoms whose shells contain electrons with spins that are not compensated by another electron of an opposing spin will have a resultant magnetic moment; this is due to the unpaired electrons. These moments tend to align (positively) with

the applied field; however, they are kept from total alignment by thermal energy. This phenomenon is referred to as paramagnetism (Kudera et al. 2016; Zhang 2018).

Cooperative Magnetism

If the atoms are in close enough contact with each other so that the electrons can be exchanged between neighbouring atoms, cooperative magnetization may occur which spontaneously aligns all atoms in a lattice and creates a synergistic and strong magnetic moment. When the spins between neighbouring atoms are aligned parallel, the material is said to be ferromagnetic. In some cases, the spins between neighbouring atoms are antiparallel and are referred to as antiferromagnetic. In antiferromagnetic materials, the resultant magnetization is small because the opposite spins cancel each other out. Lastly, if two atoms have antiparallel magnetization of unequal magnitude, the resultant magnetization remains in the direction of the stronger magnetic moment and applied field. This is referred to as ferrimagnetism.

10.1.3 Magnetic Nanoparticles

Advances in nanotechnology and molecular biology have helped to translate multifunctional nanoparticles into biomedical applications by overcoming the shortcomings related to traditional disease diagnosis and therapy. Cancer is a difficult disease to treat because of barriers in disease diagnosis and prognosis. The unique physical properties of magnetic nanoparticles (MNPs) enable them to serve as imaging probes for locating and diagnosing cancerous lesions and, simultaneously, as drug delivery vehicles that deliver therapeutic agents preferentially to those lesions. Current efforts are being carried out to combine these two properties and to develop MNP-based nano-theranostics having imaging and therapeutic functionalities that will help towards the development of personalized medicine with scope for real-time monitoring of biological responses to the therapy. So, there is always a need to summarize the existing knowledge and current progress on engineering of different MNPs and their applications from the theranostic point of view. A schematic presentation of a core-shell nanoparticle for multipurpose biomedical applications is shown in Fig. 10.3.

Iron and Iron Oxide Nanoparticles

Iron-based ferromagnetic materials have unique properties such as high magnetic moment density (which is around 220 emu/g) and are magnetically soft. Materials below the 20 nm size range show superparamagnetic behaviour. Procedures leading to monodisperse Fe nanoparticles have been well documented (Reddy et al. 2016). Different phases of iron oxide and also their related applications are shown

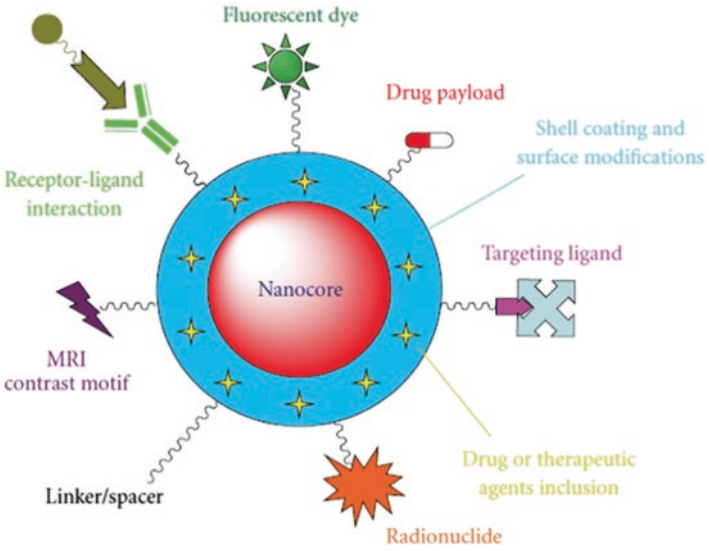

Fig. 10.3 Scheme of multifunctional nanoparticle for molecular imaging, drug delivery and therapy. Optionally functionalized and devised nanoparticles could be achieved for individualized diagnosis and treatments (Chatterjee et al. 2014; Nochehdehi et al. 2017b)

in Fig. 10.4. Nevertheless, the preparation of nanoparticles consisting of pure iron is a complicated task, because they usually contain oxide compounds, carbides and other impurities. A sample containing pure iron as nanoparticles (10.5 nm) can be obtained by evaporation of the metal in an argon atmosphere followed by deposition on a substrate (El-rouby et al. 2017). When evaporation took place in a helium atmosphere, the particle size varied in the range of 10–20 nm (Lee et al. 2018). Relatively, small (100–500 atoms) Fe nanoparticles are formed in the gas phase on laser vaporization of pure iron (Dadashi et al. 2015). The common chemical methods used for the preparations include thermal decomposition of $FeCo_5$, the particles prepared are extremely reactive, reductive decomposition of some iron (II) salts or reduction of iron (III) acetyl acetone (Wegmann and Scharr 2018). A sonochemical method for the synthesis of amorphous iron was developed (Hernández-Hernández et al. 2018).

The technique of reducing metal salts by $NaBH_4$ has been widely used to synthesize iron-containing nanoparticles in organic solvents (Huang et al. 2016). Water-soluble iron oxide nanoparticles can be obtained by wet synthesis through use of a biological coating such as oleic acid. Hydrophobic nanoparticles were synthesized by a thermal decomposition process or modified co-precipitation method through using organic solvents such as polyacrylic acid and oleic acid as capping agents (Xu et al. 2011). These nanoparticles can be used in biological applications in organic solvents stability. But aqueous stability of iron oxide nanoparticles can only occur in high molecular weight of dispersants such as poly-

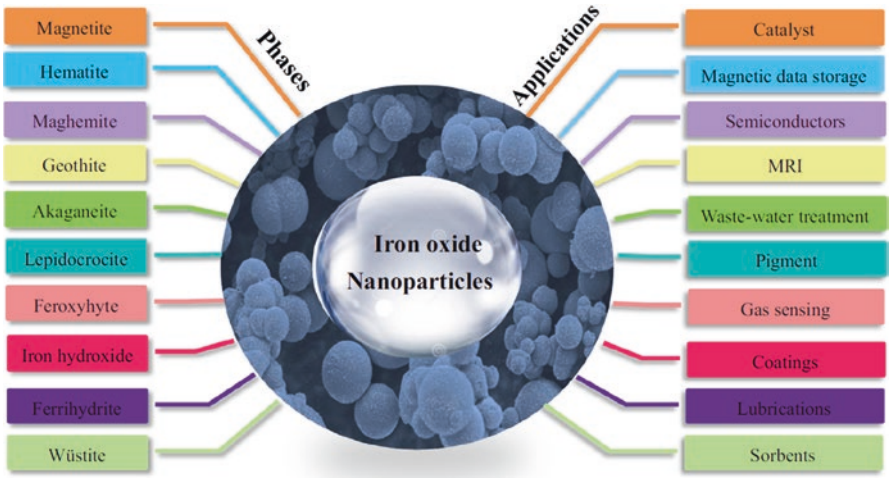

Fig. 10.4 There are various phases of multifunctional iron oxide nanoparticles (Ramimoghadam et al. 2014)

vinyl alcohol (Korpany et al. 2013). Normally, reductive synthesis of Fe nanoparticles in an aqueous solution with $NaBH_4$ yields a mixture including FeB (Miola et al. 2017; Li et al. 2017). For example, one of the most common applications of well-dispersed colloidal iron-based nanoparticles is MRI contrast enhancement and biomaterials separation. Nevertheless, the synthesis has difficulty in producing stable Fe nanoparticle dispersions, especially aqueous dispersions for potential biomedical applications. The phase composition of the obtained nanoparticles was not always reliably determined. The range of specific methods was proposed to prepare nanoparticles of defined phase composition. Thus, α-Fe nanoparticles with a body-centred cubic (bcc) lattice and an average size of ~10 nm were prepared by grinding high-purity (99.999%) Fe powder for 32 h (El-rouby et al. 2017; Landge et al. 2018; Kwon et al. 2017).

Iron oxides have received increasing attention due to their extensive applications, such as magnetic recording media, catalysts, pigments, gas sensors, optical devices and electromagnetic devices. They exist in a rich variety of structures (polymorphs) and hydration states; therefore until recently, knowledge of the structural details, thermodynamics and reactivity of iron oxides is sparse. Furthermore, physical (magnetic) and chemical properties commonly change with particle size and degree of hydration. By definition, superparamagnetic iron oxide particles are generally classified with regard to their size into superparamagnetic iron oxide particles (SPIO), displaying hydrodynamic diameters larger than 30 nm, and ultrasmall superparamagnetic iron oxide particles (USPIO), with hydrodynamic diameters smaller than 30 nm. USPIO particles are now efficient contrast agents used to enhance relaxation differences between healthy and pathological tissues, due to their high saturation magnetization, high magnetic susceptibility and low toxicity.

The biodistribution and resulting contrast of these particles are highly dependent on their synthetic route, shape and size (Klekotka et al. 2018).

Cobalt-Based Nanoparticles

Cobalt nanoparticles depending upon the synthetic route are observed in at least three crystallographic phases: typical for bulk Co HCP, ε-Co cubic (Asheesh et al. 2018; Koutsopoulos et al. 2017; Wolf et al. 2018; Maleki et al. 2018) or multiply twinned FCC-based icosahedral (Koutsopoulos et al. 2017). The conditions of synthesis have an influence on the final product structure. Often a size and phase selection was required to obtain Co nanocrystals with a specific size and shape. A popular approach is to synthesize colloidal particles by inversed micelle synthesis whereby the inverse micelles are defined as a microreactor (Vijayanandan and Balakrishnan 2018). In order to obtain stable cobalt nanoparticles with a narrow size distribution, Co(AOT) reverse micelles are used; their reduction is obtained by using $NaBH_4$ as a reducing agent. Such particles are stabilized by surfactants and are often monodispersed in size but are also unstable unless kept in a solution. Nevertheless, the chemical surface treatment by auric acid improves the stability; the cobalt nanoparticles could be stored without aggregation or oxidation for at least 1 week (Vijayanandan and Balakrishnan 2018). In many instances it is possible to obtain Co nanoparticles coated by other ligands, which can be either dispersed in a solvent or deposited on a substrate. In the latter case, self-organized monolayers having a hexagonal structure can be obtained. In some instances, with the reduction with $NaBH_4$, it is possible to obtain Co–B nanoparticles. The size, composition and structure of this kind of nanoparticles strongly depend on the concentration of the solution, pH and the mixing procedure (Ristic et al. 2017).

It is well known that the presence of oxides in magnetic materials, which form spontaneously when the metallic surface is in contact with oxygen, drastically changes the magnetic behaviour of the particles. An enhanced magneto-resistance, arising from the uniform Co resize and CoO shell thickness, has been reported (Bibi et al. 2017). This effect is caused by the strong exchange coupling between the ferromagnetic Co core and the antiferromagnetic CoO layer. However, up to now this effect has not been well understood. The ordered Co–Fe alloys are excellent soft magnetic materials with negligible magneto-crystalline anisotropy (Ramanavičius et al. 2018). The saturation magnetization of Fe–Co alloys reaches a maximum at Co content of 35 at.%; other magnetic characteristics of these metals also increase when they are mixed. Therefore, FeCo nanoparticles attract considerable attention. Thus Fe, Co and Fe–Co (20 at.%, 40 at.%, 60 at.%, 80 at.%) nanoparticles (40–51 nm) with a structure similar to the corresponding bulk phases have been prepared in a stream of hydrogen plasma (Choi et al. 2014). The Fe–Co particles reach a maximum saturation magnetization at 40 at.% of Co. The chemical reduction by $NaBH_4$ was also used for the preparation of FeCo nanoparticles (Orpe et al. 2017).

10.2 Synthetic Protocols for Magnetic Nanoparticles

Great efforts have been devoted for the preparation of MNPs due to their potential applications in many diverse fields. Different procedures to prepare two principle magnetic nanoparticles, iron oxide magnetic nanoparticles called IO-MNPs and iron-cobalt magnetic nano-alloys called IC-MNAs, will be reviewed.

10.2.1 Iron Oxide (Fe_3O_4) Magnetic Nanoparticles

Iron oxide magnetic nanoparticles have attracted the attention of researchers due to their impressive properties. Many efficient routes to attain shape-controlled, highly stable and narrow size distribution MNPs have been described in various articles. In this review we will cover different methods to synthesize IO-MNPs. Several popular methods including co-precipitation, micro-emulsion, sol-gel, thermal decomposition, solvothermal, sonochemical, microwave assisted, chemical vapour deposition, combustion synthesis, carbon arc and laser pyrolysis synthesis have been reported for the synthesis of MNPs (Takahashi et al. 2019).

Co-precipitation Technique

Co-precipitation is a basic technique to synthesize metal oxide and ferrites from aqueous solutions. Iron oxide nanoparticles and their ferrites are usually prepared in aqueous solutions. This is done by the addition of a base under inert atmosphere at room temperature or at an elevated temperature. The size, shape and composition of the MNPs completely depend on the type of salts used (e.g. chlorides, sulphates, nitrates), the reaction temperature, type of surfactant (e.g. PVP, SDS), the pH value and mixing rate. Wei et al. (2012) synthesized Fe_3O_4 nanoparticles by a co-precipitation method using sodium citrate and oleic acid as modifiers. The Fe_3O_4 nanoparticles were dried under a controlled atmosphere for 6 h at 60 °C; the ferromagnetic behaviour and magnetic saturation was around 50.61–61.36 emu. g^{-1} based on different Fe molar ratios. The magnetic saturation of Fe_3O_4 nanoparticles modified by sodium citrate and oleic acid, respectively, were 56.05 and 55.43 emu.g^{-1} which was lower than the bulk Fe_3O_4. The samples were spherical in shape with diameters around 12–15 nm. The modified Fe_3O_4 nanoparticles showed good dispersion capability in aqueous solutions which is advantageous in biomedical applications.

Rahmawati et al. (2017) synthesized Fe_3O_4 nanoparticles using co-precipitation-ultrasonic irradiation methods and also optimized the result of ultrasonic frequency and stirring rates during the synthetic process. The Fe_3O_4 nanoparticles had superparamagnetic behaviour at room temperature which is potentially useful for bioapplications. The saturation magnetization was around 25 emu.g^{-1} which

was lower than its bulk (90 emu.g^{-1}). Tang et al. (2017) synthesized Fe_3O_4 nanoparticles through a simple co-precipitation method. Iron sulphate and ammonia were used as a base to prepare nanoparticles under mechanical stirring at 95 °C. The resultant particles were vacuum-dried at 80 °C for 8 h and also annealed at 700 °C for 1 h in a nitrogen atmosphere. The Fe_3O_4 nanoparticles were polygonal in shape with a smooth surface; sizes ranged from 0.3 to 1.2 μm. In order to improve the efficiency of charge capacity for alkaline nickel-iron rechargeable batteries, nickel sulphide was coated on Fe_3O_4 nanoparticles, with thickness under 6 nm. Electrochemical specifications showed that it could effectively inhibit iron anode passivation to yield improved charge-discharge capacities as nickel-iron battery.

Sadat et al. (2014) synthesized Fe_3O_4 nanoparticles by a co-precipitation method. Ferric and ferrite salt materials were used as a base to prepare the Fe_3O_4 nanoparticles in a nitrogen environment. PAA, PS and S/Ps were coated on Fe_3O_4 nanoparticles by various methods. The structure of the nanoparticles synthesized was of inverse spinal type. In addition the thickness of the nanoparticles was determined around 6 nm. Magnetic hyperthermia behaviour of these materials was investigated in a high-frequency alternating field. It was shown that the specific absorption rate of the uncoated nanoparticles is higher than the coated ones. Figure 10.5 shows the TEM, HR-TEM and Fe-SEM images of modified Fe_3O_4 nanoparticles coated by various materials through using a co-precipitation method.

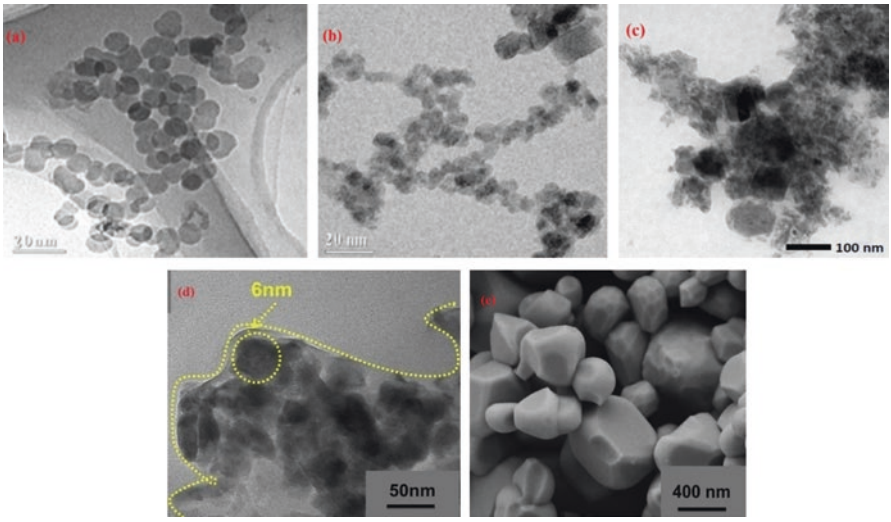

Fig. 10.5 TEM images showing the Fe_3O_4 magnetic nanoparticles which are synthesized by co-precipitation method; (**a**) modified by sodium citrate (Wei et al. 2012), (**b**) modified by oleic acid (Wei et al. 2012), (**c**) ultrasonicated (Rahmawati et al. 2017), (**d**) HR-TEM image of Fe_3O_4-NiS (Tang et al. 2017), (**e**) FE-SEM image of Fe_3O_4 (Tang et al. 2017)

Micro-emulsion Method

One of the most popular methods to synthesize uniform-sized magnetic nanoparticles is the water-in-oil micro-emulsion process. There are components, water, oil and a surfactant, in an isotropic and thermodynamically stable single-phase system. Micro-emulsions are thermodynamically stable dispersions of immiscible water phase and oil phase stabilized by the arrangement of surfactant and cosurfactant molecules at the interface. Shape and size control is one of the major advantages of this procedure. However, this method can be divided into two main categories reverse water-in-oil and normal oil-in-water. This procedure can also be used to prepare one-pot core-shell nanoparticles. Therefore, the micro-emulsion method is deemed to be one of the most cost-effective MNP production methods (Syama and Mohanan 2018).

Lu et al. (2013) synthesized Fe_3O_4 magnetic nanoparticles through a water-in-oil micro-emulsion process. They used surfactants such as n-heptane as an oil phase and n-hexanol as the cosurfactant phase. An aqueous solution of iron (II) sulphate in double distilled water was injected rapidly into the oil phase. The final solution was heated at 70 °C for 3 h under an argon atmosphere. Ultimately, the product was aged for 2 h at room temperature and dried in a vacuum oven at 80 °C for 8 h after washing with ethanol and water. The synthesized nanoparticles were of a cubic-spinal type. All samples with/without different surfactants were nearly spherical in shape with a size distribution of 10–20 nm. In addition, the magnetic saturation value of Fe_3O_4 nanoparticles in the presence of different surfactants was decreased. Nourafkan et al. (2017) developed water/mixed nonionic surfactant reverse micro-emulsions for the synthesis of iron oxide nanoparticles. Using a cosurfactant, for example, medium-chain polymers, is an effective way to reduce the surface interfacial tension of the dispersed water phase in a reverse micro-emulsion. pH values are the most important factor in reverse micro-emulsions which affect the ionizations of surface active components and the formation of different droplet sizes.

Sun et al. (2014) synthesized Fe_3O_4/polyaniline (PANI) core-shell magnetic nanoparticles by the micro-emulsion method with SDBS as a surfactant and n-pentanol as the cosurfactant. The synthesized Fe_3O_4 nanoparticles were entrapped by the PANI shell. The diameter of that core-shell was 20 nm and thickness was around 5 nm. The saturation magnetization of Fe_3O_4/PANI nanocomposites was 4.28 emu.g^{-1} with the pure Fe_3O_4 magnetic nanoparticles at 56.6 emu.g^{-1}. These nanocomposites provided good electromagnetic wave absorption performance in the range of 10.01–16.98 GHz. Figure 10.6 shows the XRD pattern and FT-IR spectra of iron oxide nanoparticles along with different surfactants.

Microwave Procedure

Most bottom-up methods for synthesizing nanomaterials include three main stages, i.e. nucleation, growth and precipitation. Amongst these steps, the growth stage depends upon the kinetics and thermodynamics of the reactions which will have a significant

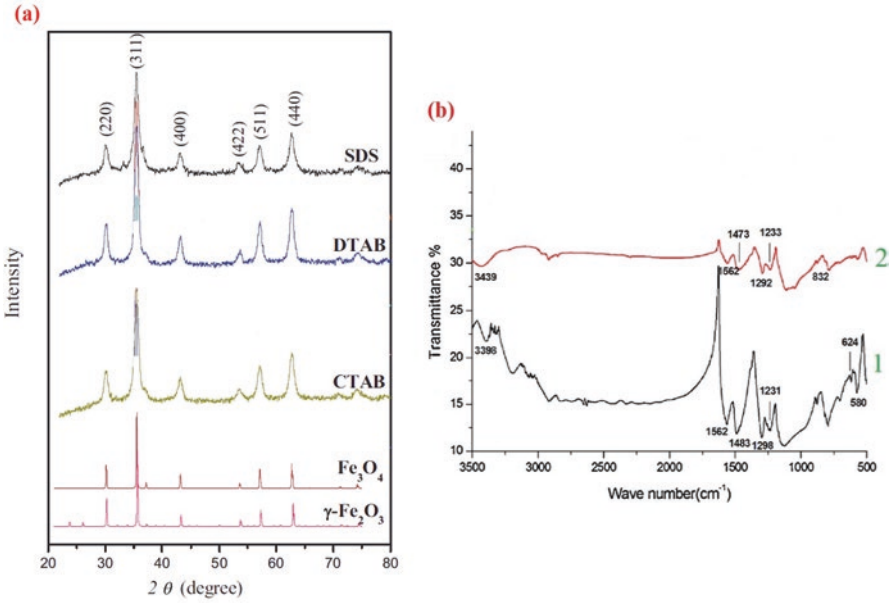

Fig. 10.6 (**a**) XRD pattern of Fe_3O_4 magnetic nanoparticles which is synthesized by co-precipitation method through using various surfactants (Lu et al. 2013) and (**b**) FT-RI spectra of (1) PANI and (2) PANI/Fe_3O_4 NPs (Sun et al. 2014)

impact on the final properties of the compounds. Microwave heating can effectively solve the problem of the non-uniformity of traditional heating caused by the formation of a very high thermal gradient in the solution. Also, the use of microwave radiation increases the speed of the initial heating process. These features have led to the use of microwaves as an effective heating and an environment-friendly method for synthesizing different nanomaterials. Figure 10.7 shows TEM images and magnetization behaviour of Fe_3O_4 nanoparticles synthesized by the microwave process.

Li et al. (2013) synthesized Fe_3O_4 magnetic nanoparticles by a microwave-solvothermal method. Ferric salt, ammonium and trisodium citrate were dissolved in ethylene glycol and then heated to 256 °C in 15 min and kept at this temperature for 2 h in a microwave. The saturation magnetization for Fe_3O_4 nanoparticles was around 30 emu.g^{-1}. As a result, the microwave-solvothermal synthesis could be suitable for large-scale synthesis of Fe_3O_4 nanoparticles. Zheng and Zhang (2010) synthesized Fe_3O_4 and $Co_2Fe_3O_4$ nanoparticles by the hydrothermal technique and electrolysis plating process, respectively. In this method, after dissolution of the metallic salt, aqua care and PEG, the resultant mixture was transferred to a Teflon-sealed autoclave and kept at 200 °C for 18 h. Wang and Chang (2012) developed a microwave method to prepare Fe_3O_4 magnetic nanoparticles. The Fe_3O_4 nanoparticles were synthesized using $FeSO_4.7H_2O$ in an alkaline medium. The spherical particles had a magnetic saturation at around 70 emu.g^{-1}.

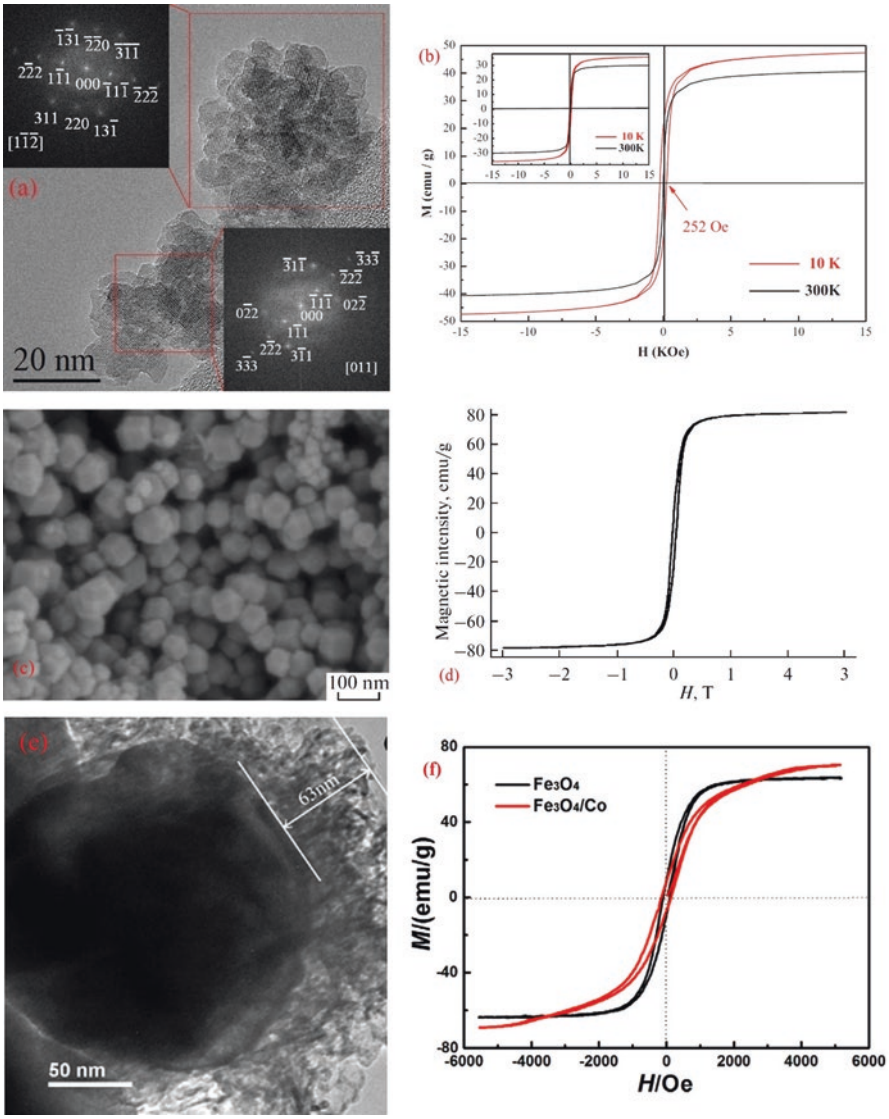

Fig. 10.7 (**a**, **b**) TEM images and hysteresis loops of Fe_3O_4 magnetic nanoparticles which are synthesized by microwave-solvothermal method (Li et al. 2013), (**c**, **d**) TEM images and magnetic hysteresis curve of Fe_3O_4 magnetic nanoparticles which are synthesized by microwave process (Zheng and Zhang 2010) and (**e**, **f**) TEM image and magnetization curve of Fe_3O_4/CO NPs (Wang and Chang 2012)

10.2.2 Iron-Cobalt (FeCo) Magnetic Nano-alloys

Over the past decade, design, synthesis, characterization and implementation of novel ferromagnetic nano-alloys have been considered by many researchers around the world. FeCo-based alloys have specifically gained interest due to elevated magnetization along with a high Curie temperature (~900 °C), high saturation magnetizations, high permeability, low magnetic losses at high frequencies and being relatively strong and good mechanical strength (Kudera et al. 2016; Shavandi et al. 2018; Lam et al. 2018; Çelik and Fırat 2018; Albaaji et al. 2017). Since the first report of the synthesis of FeCo as a soft magnetic material by Elmen in 1929 (El-Gendy 2018), there has been considerable research done to understand the physics of their magnetism and how to improve their properties (Li et al. 2019a; Klencsár et al. 2016; Huo et al. 2018; Codescu et al. 2019; Adamiano et al. 2018). The ferromagnetic alloys have been prepared by several methods, including thermal decomposition, sonochemical reduction, arc discharge and laser pyrolysis, the polyol process and aqueous reduction by borohydride derivatives (Wang et al. 2019a; Kandapallil et al. 2015; Yang et al. 2018; Fan et al. 2015). These synthesis techniques have produced several shapes including spheres, cubes, dice and wires. Aqueous reduction by borohydride has been used to produce monometallic nanoparticles of CoB and FeB (Wang et al. 2017; Barbosa et al. 2019; Zare et al. 2017; Yang et al. 2016, 2017; Cai et al. 2016). Recently, it was shown that by using a capping agent, such as sodium citrate, they can eliminate the formation of FeB/Fe_2B nanoparticles to form elemental α-Fe. As mentioned above, there are different methods and techniques to synthesize the iron-cobalt nano-alloys. We will review some of the well-known methods to synthesize FeCo nano-alloys.

Polyol Procedure

Polyol synthesis of nanoparticles is a liquid phase synthesis method involving multi-basic alcohols with high boiling points. Polyols have a high degree of biodegradability and biocompatibility and are also known as green solvents. Different types of polyols for reducing metal salts through with presence of hydroxyl sites are shown in Fig. 10.8. In general, polyols provide a variety of advantages for the synthesis of nanoparticles. The useful features of polyols can be related to their degradation properties that allow for the direct synthesis of metal nanoparticles.

Zehni et al. (2014) synthesized soft magnetic $Fe_{55}Co_{45}$ alloy nanoparticles by using the polyol reduction process. In this method, the metallic salt was dissolved in diethylene glycol (DEG). The $FeCo_2O_4$ was formed when the reaction temperature was increased to 873 K for 4 h under argon gas atmosphere. The nanoparticles had a spinal structure with a cubic morphology. In addition, the magnetic saturation was completely dependent on the annealing temperature. Kodama and Shinoda (2007) synthesized FeCo nano-alloys by using the polyol method by dissolving the metallic salt in ethylene glycol using PVP as a surfactant. Abbas et al. (2013) syn-

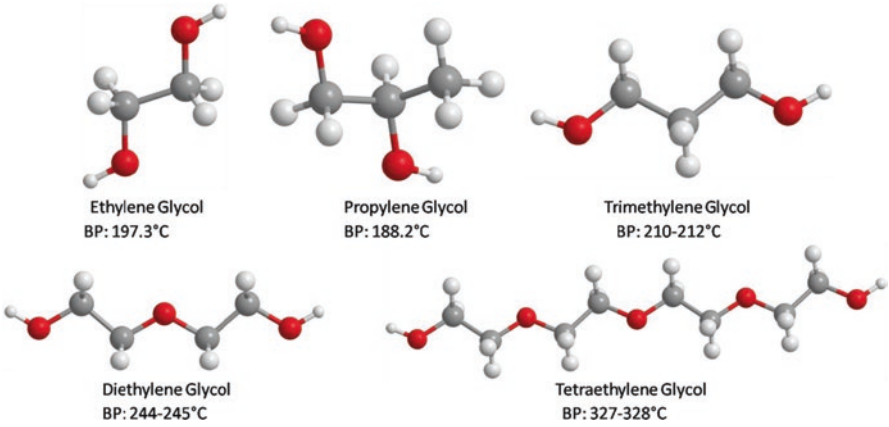

Fig. 10.8 Effect of hydroxyl sites through using various polyols to reduce the metal salts (Kudera et al. 2016)

thesized high magnetization air-stable spherical FeCo nanoparticles with an average size of 10 nm. The magnetic saturation of the annealed FeCo nanoparticles was around 230 emu.g^{-1}.

Joseyphus et al. (2007) designed the synthesis of cobalt and its alloys via a polyol process. The metallic salt was dissolved in trimethylene glycol and heated at a rate of 15 °C/min to the boiling point of polyol. Reaction and annealing time, temperature, concentration of solvent and surfactants are the main factors to control the size and shape of nanoparticles prepared by the polyol method. Figure 10.9 shows characterization of FeCo nanoparticles synthesized by the polyol method.

Sol-Gel Method

The sol-gel process is a wet chemical method used to prepare a variety of nanostructures materials. The molecular precursor is dissolved in water or alcohol, then heated and stirred. The colloid solid hydrolysis (sol) acts as a precursor for an integrated network (gel) of particles. There is good control over the chemical composition of the products because the low reaction temperature can be used in this process. Braga et al. (2015) synthesized FeCo air-stable nanocrystals by the sol-gel method using a 1:1 concentration of Fe and Co. There were two different solutions: the first one contained the iron nitrate, and the second one contained the cobalt nitrate for this reaction which was maintained under constant thermal agitation at 100 °C to obtain the uniform gel.

Lobo et al. (2015) synthesized spinal $FeCo_2O_4$ nanoparticles by using the sol-gel method. Iron nitrate and cobalt acetate were used in the formation of the gel. The gel dried at vacuum oven at 120 °C and also pre-synthesized at 300 °C for 4 h. Ultimately, the powder was aged at 900 °C for 12 h. The saturation magnetization of the spinal $FeCo_2O_4$ was 92.28 emu.g^{-1}, which shows the ferromagnetic property

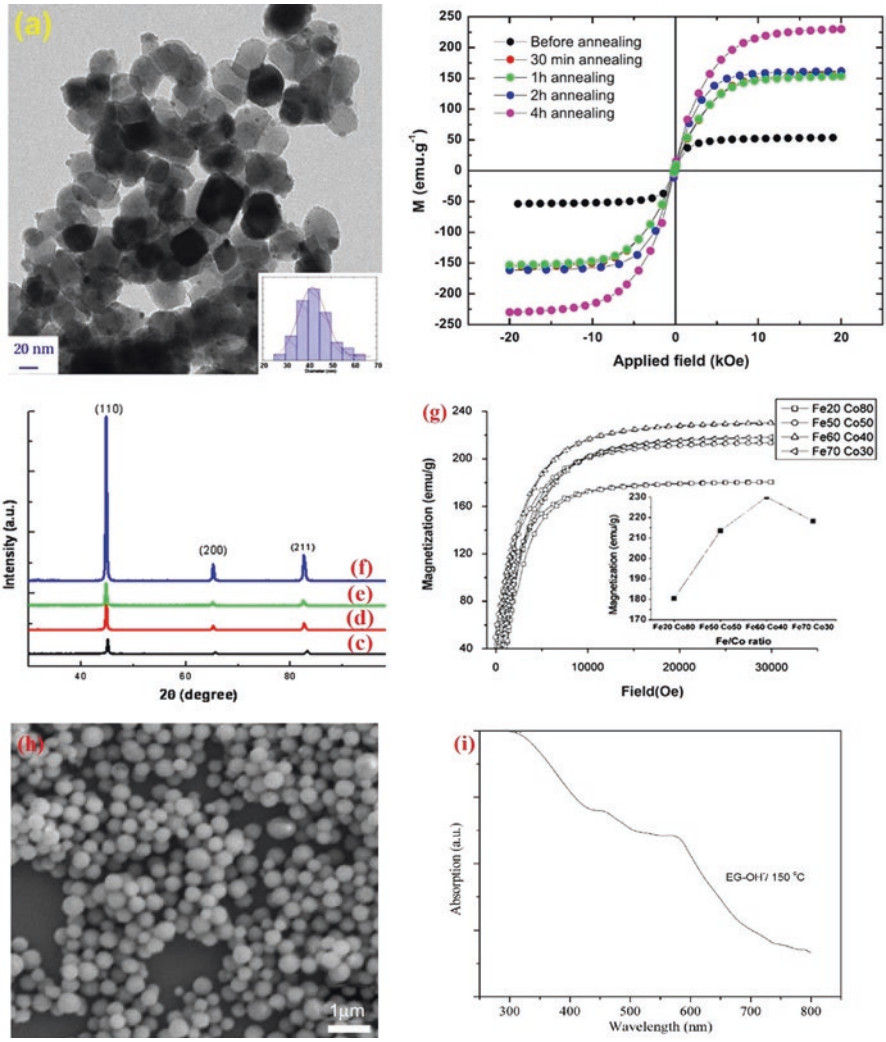

Fig. 10.9 (**a**) HR-TEM image of FeCo nanoparticles which are annealed for 30 min at 873 K (Zehni et al. 2014), (**b**) hysteresis loops of FeCo nanoparticles which are annealed at different temperatures (Chokprasombat and Pinitsoontorn 2016); XRD pattern of FeCo nanoparticles which are synthesized and annealed at 873 K (**c**) $Fe_{20}Co_{80}$ (Kodama and Shinoda 2007), (**d**) $Fe_{50}Co_{50}$ (Kodama and Shinoda 2007), (**e**) $Fe_{60}Co_{40}$ (Kodama and Shinoda 2007), (**f**) $Fe_{70}Co_{30}$ (Kodama and Shinoda 2007); (**g**) magnetization of FeCo nanoparticles with various atomic ratios (Kodama and Shinoda 2007), (**h**) SEM image of FeCo nanoparticles which are synthesized at 473 K through polyol method (Abbas et al. 2013), (**i**) UV visible spectra of Co-EG-[OH] (Abbas et al. 2013)

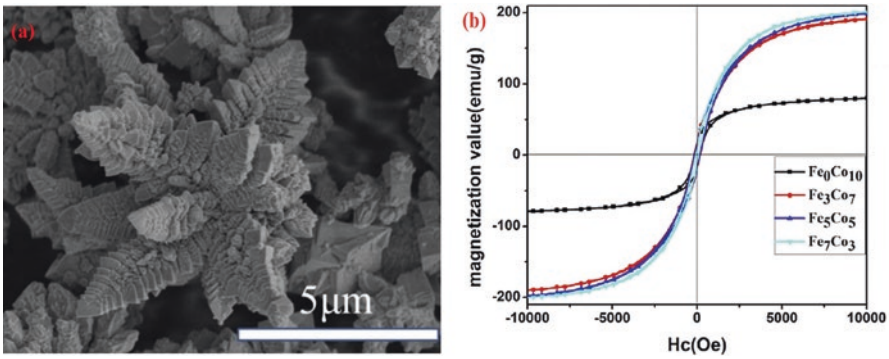

Fig. 10.10 (**a**) SEM image of $Fe_{30}Co_{70}$ nanoflowers which are synthesized by sol-gel method (Cheng et al. 2017), (**b**) magnetic curve and hysteresis loops of different FeCo nanoparticles (Cheng et al. 2017)

of the material. Nautiyal and Motin Seikh (2015) synthesized Fe–Co nanoparticles using iron and cobalt nitrate in ethylene diamine and citric acid. The reaction mixture solution was stirred at 60 °C for 3 h and also evaporated at 100 °C to form a gel. The gel mixture was dried at 150 °C for 12 h; then the resulting powder was aged at 250 °C for 3 h. Iron-cobalt nanoparticles were synthesized by a simple liquid thermal reduction method (Cheng et al. 2017). Particles with flowerlike morphology (Fig. 10.10a) were observed with sizes in the 1.6–2.8 μm range with magnetic properties shown in Fig. 10.10b.

10.3 Surface Modification and Coating Process of Magnetic Nanoparticles

Core-shell nanoparticles are hybrid systems, with various types of cores, as well as different types of shells. Each core and shell can have properties such as metal conductivity, semi-conductivity and magnetism. Any compilation of core and shell is possible. In order to improve chemical, physical, mechanical properties and biocompatibility, the surface can be modified with a wide range of compounds. Also, coating with different materials such as polymeric, ceramic or metallic compounds which is related to their applications is possible. The interesting aspect of these systems is the possibility to protect the core from the surrounding chemical environment. There are different procedures for coating nanoparticles from chemical methods such as co-precipitation to thermal-vapour decomposition and ball milling.

10.3.1 Iron-Based Core-Shell Magnetic Nanoparticles

There have been many synthetic procedures developed to prepare iron oxide core-shell magnetic nanoparticles. The shell can be compounds of polymers, ceramics and metallic compounds related to specified applications. Venkateswarlu et al. (2015) synthesized Fe_3O_4-Ag core-shell nanoparticles by a novel eco-friendly method. In this method, aqueous *V. Viniferous* steam extract which had biological functional groups such as carbohydrates and polyphenols was used as a reducing agent. The mixture of the reducing agent and metal salt was maintained at 343 K; silver nitrate was then added to the samples. The resulting powder was dried in a vacuum oven at 363 K. The Fe_3O_4-Ag nanoparticles had a spherical morphology with ferromagnetic behaviour around 15 $emug^{-1}$. Ganjali et al. (2013) synthesized the $Fe_3O_4@SiO_2$ core-shell magnetic nanoparticles by a co-precipitation method in an alkali solution. Two iron salts were dissolved in deionized water. Then the solution was added to the ammonium solution under nitrogen gas at 80 °C for 2 h to obtain small, uniform particles. The resultant powder was aged at 100 °C in vacuum for 24 h and then added to the methanol. Tetraethyl orthosilicate was added to the reaction vessel and continuously stirred at 40 °C for 24 h. The silica-coated iron oxide nanoparticles were dried in vacuum at 60 °C for 24 h.

Liu et al. (2013) synthesized Fe_3O_4-Au core-shell nanoparticles through a co-precipitation method as carriers of the primary antibody of carbohydrate antigen. In order to modify the surface, APTES was added to the Fe_3O_4 nanoparticles dispersed in ethanol followed by stirring for 7 h at room temperature. The as-prepared APTES-coated Fe_3O_4 MNPs were used as seeds. Au NPs were coated by reducing $HAuCl_4$ in the presence of sodium citrate. The dark purple nanoparticles were obtained by separation with a magnetic field.

Fe_3O_4-chitosan core-shell nanoparticles were prepared by an alkaline precipitation method using ferrous chloride as the source (Patil et al. 2014). The resultant Fe_3O_4 nanoparticles were dried at 100 °C. The chitosan was coated to the Fe_3O_4 nanoparticles by an ultrasonication method. Fe_3O_4 nanoparticles were dispersed in distilled water, with chitosan dissolved in acetic acid. The mixture was ultrasonicated for 30 min and then dried at 50 °C. The morphology of the pure Fe_3O_4 and chitosan-coated Fe_3O_4 nanoparticles was spherical, with particle sizes of around 20–23 nm. Both samples had a hysteresis curve, and the magnetic saturation of the pure Fe_3O_4 and chitosan-coated nanoparticles, respectively, was around 55 $emu.g^{-1}$ to 48 $emu.g^{-1}$, which is typical of superparamagnetic behaviour. Wang and Chang (2012) synthesized Fe_3O_4-Co core-shell nanoparticles by a hydrothermal technique and an electroless plating process. The hydrothermal technique emphasized ethylene glycol as a solvent. The final solution was placed in a Teflon-sealed autoclave at 200 °C for 18 h, followed by drying at 60 °C. The Fe_3O_4 powder was then dispersed in sodium hydroxide solution and the miscible liquid was added in an electroless plating bath.

10.3.2 Cobalt-Based Core-Shell Magnetic Nanoparticles

In recent years, in order to expand the application areas of nanoparticles in medicine especially in drug delivery system and therapeutic techniques, cobalt-based core-shell magnetic nanoparticles (CCS-MNPs) were developed. These nanoparticles have high magnetic saturation and biocompatibility. Xu et al. (2013) synthesized FeCo@C core-shell magnetic nanoparticles by decomposition of benzene through a sol-gel process and hydrogen reduction. In this case, the metallic salt with different atomic ratios and citric acid was dissolved in ethanol. The temperature of the solution was increased to 150 °C to prepare the gel. This gel was heated in air at 500 °C for 4 h. The powder was placed inside tat 500 °C for 4 h, followed by passing benzene under argon flow. The diameter of the FeCo@C was around 100 nm, with magnetic saturation around 197 emu.g^{-1}. Lu et al. (2007) synthesized FeCo@SiO_2 core-shell magnetic nanoparticles using a wet chemical procedure. The metallic salt was dissolved in distilled water. Sodium silicate was then added as a suspension at 80 °C to perform the hydrolyzation of the silicate.

The resulting powder was dried at 100 °C and then reduced by hydrogen gas in a quartz furnace. Particle sizes of the FeCo@SiO_2 nanocomposite were between 30 and 70 nm, with saturation magnetization at around 200 emu.g^{-1}. FeCo@SnO_2 was synthesized through a chemical procedure (Wang et al. 2016). The FeCo nanoparticles were synthesized by a co-precipitation method through dissolving the metallic salt in distilled water at 110 °C for 2 h in the presence of hydrazine hydrate. In order to coat the FeCo nanoparticles, the $SnCl_2$ was added in an ammonia propanediol and deionized water solution. The size of the FeCo core was around 200 nm, while the thickness of the SnO_2 was around 20–30 nm. The magnetic saturation of the FeCo nanoparticles and FeCo@SnO_2 nanocomposites was detected around 172 and 36 emu.g^{-1}, respectively.

Wen et al. (2013) synthesized FeCo@Ru nanoflowers using a solvothermal process. The metallic salt containing the Fe^{3+}, Co^{2+} and Ru^{3+} was dissolved in ethyl alcohol followed by the addition of ethylene glycol. This solution was placed in an autoclave at 180 °C for 15 h with a temperature rate of 1 °C.min^{-1}. The magnetic saturation of the FeCo@Ru nanoflowers was around 155 emu.g^{-1}, showing superparamagnetic behaviour. Chokprasombat and Pinitsoontorn (2016) synthesized FeCoNi ternary alloys with different atomic ratio through a chemical reduction process. The spherical FeCoNi particles had a diameter around 60 nm. As a comparison, a brief overview of the methods, materials, applications and properties of the mentioned nanoparticles is shown in Table 10.1.

Table 10.1 Detailed information of Fe_3O_4 and FeCo nanoparticles

Type of nanoparticles	Synthesis procedure	Particle size (nm)	Type of coating	Particle shape	Magnetic saturation (emu.g^{-1})	Applications
Fe_3O_4						
FeCo	Co-precipitation	5–60	SnO_2 Au Cu Ag Carbon (grapheme) Ni Co Ru Mn Mg ZnO ZrO_2 Pu PEG PVP PLA PEI Chitosan Hydroxyapatite SiO_2	Cubic	20–140	Separation Immunoassay Drug delivery systems (to eyes and brain) Nanodrug carriers Gene transmitter Magnetic resonance imaging (MRI) Hyperthermia Analytical tools, nanomaterials and nano-devices Regulatory and toxicological issues Optical devices Nano-biosensors Data storage Catalyst Gas sensor, agriculture applications, packaging industry Environmental remediation applications
	Chemical reduction	3–80			20–160	
	Sol-gel	20–200			10–130	
	Thermal decomposition	30–120			30–140	
	Chemical and vapour deposition	5–50		Spherical	20–80	
	Physical vapour deposition	4–60		Tri-angle	10–80	
	Laser deposition	3–40			20–80	
	Polyol	1–80			30–200	
	Micro-emulsion	4–20			30–160	
	Microwave	3–70		Flowered	30–180	
	Solvothermal	5–50			30–180	

Wei et al. (2012), Rahmawati et al. (2017), Tang et al. (2017), Sadat et al. (2014), Syama and Mohanan (2018), Lu et al. (2007, 2013), Nourafkan et al. (2017), Sun et al. (2014), Li et al. (2013, 2019a), Zheng and Zhang (2010), Wang and Chang (2012), Wang et al. (2016, 2017, 2019a), Shavandi et al. (2018), Lam et al. (2018), Çelik and Fırat (2018), Albaaji et al. (2017), El-Gendy (2018), Klencsár et al. (2016), Huo et al. (2018), Codescu et al. (2019), Adamiano et al. (2018), Kandapallil et al. (2015), Yang et al. (2016, 2017, 2018), Fan et al. (2015), Barbosa et al. (2019), Zare et al. (2017), Cai et al. (2016), Zehni et al. (2014), Kodama and Shinoda (2007), Abbas et al. (2013), Joseyphus et al. (2007), Braga et al. (2015), Lobo et al. (2015), Nautiyal and Motin Seikh (2015), Cheng et al. (2017), Venkateswarlu et al. (2015), Ganjali et al. (2013), Liu et al. (2013), Patil et al. (2014), Xu et al. (2013), Wen et al. (2013), Chokprasombat and Pinitsoontorn (2016)

10.4 Biomedical Applications of Magnetic Nanoparticles

Magnetic nanoparticles have been proposed for biomedical applications for several years (Mohammed et al. 2017; Kondo 2018; Alonso et al. 2018). Examples include targeted drug delivery, hyperthermic treatment for malignant cells and magnetic resonance imaging (Kondo 2018; Alonso et al. 2018; Tapeinos 2018; Baker 2018). There are three reasons why magnetic nanoparticles are useful in biomedical applications. Living organisms are built of cells that are typically 10 μm in diameter. However, the cell parts are much smaller and in the submicron size domain (Gubin 2009). Magnetic nanoparticles have controllable sizes ranging from a few nanometres up to tens of nanometres, which places them at dimensions that are smaller than those of a cell (10–100 μm), or comparable to the size of a virus (20–450 nm), a protein (5–50 nm) or a gene (2 nm wide and 10–100 nm length), is the first advantage of BMANPs in medicine. This means that they can "get close" to a biological entity of interest. This simple size comparison gives an idea of using nanoparticles as very small probes that would allow us to spy at the cellular machinery without introducing too much interference. Indeed, they can be coated with biological molecules to make them interact with or bind to a biological entity, thereby providing a controllable means of "tagging" or addressing it.

Secondly, if nanoparticles are magnetic, they can be manipulated by an external magnetic field gradient. This "action at a distance" combined with the intrinsic penetrability of magnetic fields into human tissue opens up many applications involving the transport and immobilization of magnetic nanoparticles or of magnetically tagged biological entities. In this way, they can be made to deliver a package, such as an anticancer drug, to a targeted region of the body, such as a tumour.

Thirdly magnetic nanoparticles can be made to resonantly respond to a time-varying magnetic field, with advantageous results related to the transfer of energy from the exciting field to the nanoparticle. For example, the particle can be made to heat up, which leads to their use as hyperthermia agents, delivering toxic amounts of thermal energy to targeted bodies such as tumours, or as chemotherapy and radiotherapy enhancement agents, where a moderate degree of tissue warming results in more effective malignant cell destruction. These, and many other potential applications, are made available in biomedicine as a result of the special physical properties of magnetic nanoparticles. Understanding of biological processes on the nanoscale level is a strong driving force behind the development of nanotechnology (Gubin 2009; Tartaj et al. 2016).

For biomedical applications, magnetic nanoparticles must (1) have a good thermal stability, (2) have a large magnetic moment, (3) be biocompatible, (4) be able to form stable dispersion so the particles could be transported in living system and (5) respond well to AC magnetic fields. Magnetic nanoparticles can be a promising tool for several applications in vitro and in vivo. In medicine, many applications were investigated for diagnostics and therapy, and some practical approaches were chosen. Magnetic immunobeads, magnetic streptavidin, DNA isolation, cell immune magnetic separation, magnetic resonance imaging, magnetic targeted delivery of therapeutics or magnetically induced hyperthermia are approaches of

particular clinical relevance. Investigations on applicable particles induced a variability of micro- and nanostructures with different materials, sizes and specific surface chemistry. Nanoparticles for medicine are useful for therapy, imaging and diagnostics of cancer and other diseases leading an entrapped or bound therapeutic or diagnostic target material to the area of interest, e.g. a tumour. The destination – targeted delivery – may be found by physical forces (magnetic) or with surface-bound antibodies (cell-/tissue-specific) (Khanna et al. 2018). Some present applications of nanomaterials in biology and medicine are fluorescent biological labels (Cotin et al. 2018; Pašukonienė et al. 2014; Ahamed et al. 2016), drug and gene delivery (De-La-Cuesta et al. 2018; Marciello et al. 2016), bio-detection of pathogens (Kumar et al. 2017), detection of proteins (Epherre et al. 2017), probing of DNA structure (Ansari et al. 2017), tissue engineering (Atukorale et al. 2017), tumour destruction via heating (hyperthermia) (Salunkhe et al. 2016), separation and purification of biological molecules and cells (Clemons et al. 2019), MRI contrast enhancement (Fatima and Kim 2018) and phagokinetic studies (Idowu et al. 2018). As mentioned above, nanomaterials are suitable for biotagging or labelling because they are the same size as proteins. Another feature to use nanoparticles as biological tags is their biosusceptibility. Examples of biological coatings may include antibodies, biopolymers like collagen (Miola et al. 2017) or molecule monolayers (amino acids, sugars) that make the nanoparticles biocompatible (Lee et al. 2018; Landge et al. 2018; Kwon et al. 2017).

Nanoparticles can be used as a convenient surface for molecular assembly and may be composed of inorganic or polymer materials. It can also be in the form of a nanovesicle surrounded by a membrane or a layer. The shape is not automatically spherical but sometimes cylindrical or platelike. The size and size distribution might be important in some cases, for example, if penetration through a pore structure of a cellular membrane is required. The size and size distribution are extremely critical when quantum-sized effects are used to control material properties. A tight control of the average particle size and a narrow distribution of sizes allow for the creation of efficient florescent probes that emit narrow light in a very wide range of wavelengths. This helps creating biomarkers with many well-distinguished colours. The core itself might have several layers with multifunctionality. For example, by combining magnetic and luminescent layers, one can both detect and manipulate the particles (Djordjevic et al. 2018). Biomedical applications of different nanoparticles are shown in Table 10.2 (Chatterjee et al. 2014).

10.4.1 Magnetic Nanoparticles in Bioimaging

Magnetic Resonance Imaging

MR imaging, one of the most powerful non-invasive imaging methods utilized in clinical medicine, is based on the relaxation of protons in tissues (Guleria et al. 2018). Upon accumulation in tissues, superparamagnetic iron oxide magnetic

Table 10.2 Biomedical applications of different nanoparticles (Chatterjee et al. 2014)

Core-shell nanoparticles	Surface modification	Application
Fe_3O_4/SiO_2	Fluorescein isothiocyanate dye, chelated	MRI, amperometric sensor
Fe_3O_4/PAH/Au		
Fe_3O_4/chitosan or oleic acid	Haemoglobin for H_2O_2 detection/enzymes, nucleotides	MRI, optical imaging and drug delivery
Fe_3O_4/silica/Au		
Fe_2O_3/PEG or PEI		MRI
Fe_2O_3/2methacryloyloxy ethyl		X-ray
Fe_2O_3/SiO_2/Au	PEG, amino acid, FTIC, antibody conjugation	MRI, biolabelling, optical imaging, drug delivery
$Fe_3O_4/CaCo_3$/PMMA/MnO	PEG, glucuronic acid	MRI, cell labelling
Fe oxide or Fe_3O_4/Au	DNA ligase enzyme	Piezometric and optic sensor
Fe_3O_4 embedded in poly(D-lactid)/PLA/PVP		MRI, ultrasound
Fe/CNP	Poly(acrylic acid) (PAA), polyvinyl pyrrolidone (PVP), poly(2-acetoxyethyl methacrylate) (PAEMA)	MRI
FeCo/Au	PNA oligomers	MRI, optical sensor
FeCo/C	PAA	MRI, hyperthermia cancer treatment
FeCo	Hydroxyapatite	MRI, hyperthermia cancer treatment

nanoparticles enhance proton relaxation of specific tissues compared with that in surrounding tissues, serving as an MR contrast agent (Delgado-Rosales et al. 2018). In vivo MR imaging applications, SIONPs should have a long half-life time in blood circulation for the improved efficiency of detection, diagnosis and therapeutic management of solid tumours. Because opsonin plasma proteins are capable of interacting with plasma cell receptors on monocytes and macrophages, opsonin-absorbed SIONPs will be quickly cleaned by circulating monocytes or fixed macrophages through phagocytosis, leading to the elimination of SIONPs from blood circulation. The smaller the particle, the more neutral and hydrophilic its surface, and the longer its plasma half-life (Li et al. 2019b). Therefore, the surface of SIONPs has been modified with hydrophilic polymers to prevent absorption of the circulating plasma proteins. The use of contrast agents and tracers in medical imaging has a long history (Schröfel et al. 2014; Muthuraman et al. 2018; Hemalatha et al. 2018). Known collectively as imaging contrast agents, these molecules possess physical characteristics that increase the strength of the signal coming out of the body. The contrast agents containing the element gadolinium (or iron), for example, do so by altering the magnetic field in the body, which boosts the strength (or reduce) of the MRI

signal. They provide important information for diagnosis and therapy, but for some desired applications, a higher resolution is required than can be obtained using the currently available medical imaging techniques.

Computed Tomography

Computed tomography provides a good imaging modality for studying anatomical details as against positron emission tomography (PET) and other modalities which focus on metabolic pathways using X-ray absorption spectra as a detection signal. Gamma rays used in PET analysis have lower energy levels and lower penetration power than X-ray used in computed tomography. Thus, high-resolution CT is most constructive in tracking the site and loci of some metabolic event or analysing its histological impact as X-ray emissions are differentially absorbed by tissues according to their X-ray attenuation coefficient, which gives a visual spectrum for image reconstruction (Delgado-Rosales et al. 2018). To achieve high resolution, several nanoparticle-based contrasting agents based on iodine, barium, barium sulphate, etc. are in use, which selectively highlight the tissue of interest during computed tomography analysis.

Conventional CT contrast agents have generally high renal toxicity and suffer from low imaging time, because of rapid renal clearance. Low molecular weight nanoparticle systems comprising of gold/iron oxide core-shell nanoparticles have shown great potential to be used as computed tomography contrasting agents, for their stability and optimum residence time in the tissues and versatility in multifunctional imaging mode. Recent developments include using multimodal nanoparticles contrasting agents especially using electron-dense elements such as iodine (Wilk 2019; Wang et al. 2019b; Khademi et al. 2018; Sasaya et al. 2018; Lin et al. 2018) or bismuth which form well-defined dispersion spectra when impinged by electromagnetic waves. Biocompatible, dual-mode contrasting core-shell nanoparticle-based agents have been reported, recently. Molecules are the most common radiotracers used for dual imaging purposes in PET/CT (Mallak et al. 2018). Nanoparticles having a superparamagnetic iron core cross-linked with dextrin forming the corona-bonded molecules empower them to work for PET, CT and MRI.

10.4.2 Magnetic Nanoparticles in Targeted Drug Delivery

Drug delivery remains a challenge in the management of cancer and another illness. The focus is on targeted cancer therapy. The newer approaches to cancer treatment not only supplement the conventional chemotherapy and radiotherapy but also prevent damage to normal tissues and prevent drug resistance. Innovative cancer therapies are based on current concepts of molecular biology of cancer. These include antiangiogenic agents, immunotherapy, bacterial agents, viral

oncolysis, targeting of cyclic-dependent kinases and tyrosine kinase receptors, antisense approaches, gene therapy and combination of various methods. Important methods of immunotherapy in cancer involve use of cytokines, monoclonal antibodies, cancer vaccines and immunogene therapy (Mallak et al. 2018). The innovative pharmaceutical treatments obviously require novel modern methods of administration. The possibility of using ferro-fluids for drug localization in blood vessels and in hollow organs is a contemporary task for drug development (Dhas et al. 2018). Pure magnetic particles are not stable in water-based solutions and suspensions; therefore, they cannot be used for medical application without biocompatible coating. The choice of polymers for magnetic nanoparticles coating to prevent them from adhering toxicity occurred to be not an easy one-step task, because these compositions should satisfy both the requirements of biocompatibility and biodegradability.

During the past two decades, research into hydrogel delivery systems has focused primarily on systems containing polyacrylic acid (PAA) backbones. PAA hydrogels are known for their superabsorbency and ability to form extended polymer networks through hydrogen bonding. In addition, they are excellent bioadhesives, which mean that they can adhere to mucosal linings within the gastrointestinal tract for extended periods, releasing their encapsulated medications slowly over time. Today different nano-systems used in controlled drug delivery are known; these are shown in Fig. 10.11.

10.4.3 Magnetic Nanoparticles in Targeted Gene Therapy

A therapeutic tissue targeting of medicines in the organism with magnetic field force is a doubtful thing. However, one delivery system occurred to be appropriate for the targeting delivery purpose; it is sterically stabilized liposomes platform. In general, magnetic liposomes are also included when speaking about magnetic carriers. Sterically stabilized (polyethylene glycol-containing) liposomes or PEG-liposomes technology has been used to overcome some of the barriers of drug delivery. In general, liposomes consist of phosphatidylcholine, cholesterol (Chol), phosphatidylethanolamine and PEG with different molecular weight from 300 to 2000 covalently attached to PEA. They can be used as carriers of magnetic nanoparticles as well as for transportation of antitumour chemotherapeutic agents. Encapsulating anticancer drugs in liposomes (magnetic or nonmagnetic) enables drug delivery to tumour tissues and prevents damage to the normal surrounding tissues. The advantages of administration of sterically stabilized liposomal agents over simple drug forms are well proven in multiple laboratories (Mohanta et al. 2018; Chamundeeswari et al. 2018; Dey et al. 2017; Jindal 2017) and clinic assays (Mankamna Kumari et al. 2018). Liposomes provide time-release of the encapsulated medicines after a single administration together with significant diminishing of systemic toxicity.

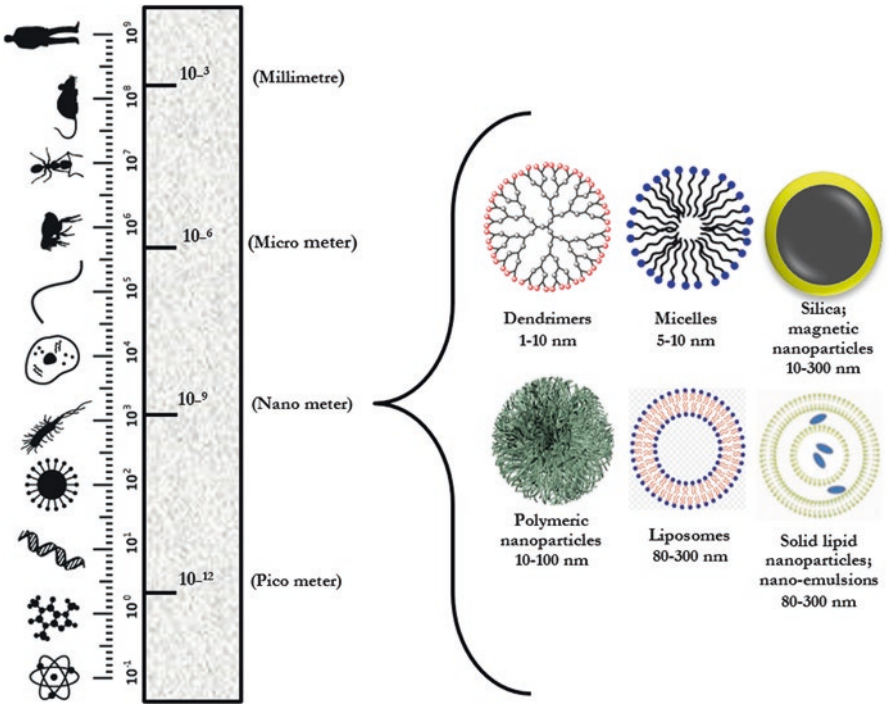

Fig. 10.11 Application of various magnetic nanoparticle systems to targeted drug delivery techniques for comparing with different objects due (Chen et al. 2018)

10.4.4 Magnetic Nanoparticles in Interaction of Nanoparticles with DNA and RNA

The interaction of nanoparticles with human cell has been a topic of profound interest among researchers, as they are thought to hold the key for future developments in the fields of biodiagnostic and therapeutic, amongst other fields. The range of nanoparticles between 50 and 200 nm has been deemed most effective for uptake in cells, and this has opened new avenues of applications (Gehr 2018). Gold nanoparticles are most commonly used for the detection of DNA, and spectroscopic and electrophoretic technique has been applied to evaluate the interaction of Au with calf thymus DNA (Tian et al. 2019). The use of gold nanoparticles has been prevalent as they can easily be synthesized in a relatively pure and monodispersed form. Assah et al. (2018) studied the effect of pH on the assembly of ss-DNA-functionalized Au nanoparticles. Since the isoelectric point (IP) of ss-DNA is between pH 4 and 4.5, they are negatively charged above this pH and are easily conjugated with Au nanoparticle. They used this Au-ss DNA assembly for single-base mismatch detection and successfully applied it to detect 12 point mutations derived from human p53 gene. This methodol-

ogy is unique in the sense that it neither requires complex DNA modifications nor signal amplifications; however the only limitation is that it requires two individual reactions for comparison between a wild-type sequence and a mutant sequence.

The core-shell nanoparticles can also be used for the inhibition of DNA hybridization (Tian et al. 2019). It has been found that surface modification of Ag/Pt core-shell nanoparticles by thiol-modified oligonucleotides successfully reduces the nonspecific interaction between DNA and nanoparticles, by increasing the Pt particle size. Similarly, gold-coated magnetic NPs (Co and Fe_3O_4) core-shell nanoparticles and their interaction with thiolated DNA were also studied (Badiki et al. 2017). Reduction of Au salt over preformed magnetic cores resulted in composite-type NPs. Core-shell NPs with Co gave Co–Au alloy-type deposition shell, while in Fe_3O_4 core NP has distinct magnetite and gold phases. In general, functionalized magnetic NPs with Au shell facilitate thiol-mediated conjugation of DNA on nanoparticle's surface. Mesoporous silica nanoparticle containing Fe_3O_4 inner core and silica shell has been prepared to study the DNA adsorption and desorption process, and it has the added advantage of separation by the application of external magnetic field (Tian et al. 2018).

10.4.5 Magnetic Nanoparticles in Cancer Diagnosis and Treatment Via Hyperthermia Method

A common failure in targeted systems is due to the opsonization of the particles on entry into the bloodstream, rendering the particles recognizable by the body's major defence system. In some cases, the combination of gene therapy with effects of hyperthermia may be possible. Heat-induced therapeutic gene expression is highly desired for gene therapy to minimize side effects. Furthermore, if the gene expression is triggered by heat stress, combined therapeutic effects of hyperthermia and gene therapy may be possible (Takahashi et al. 2019). Hyperthermia therapy is a type of cancer treatment in which the body tissue is exposed to high temperature of 42 °C or higher, which is found to be more harmful to cancer cells than to normal healthy cells. Mild hyperthermia is performed at 41–46 °C to simulate the immune response for non-specific immunotherapy of cancers, while thermoablation is performed at 46–56 °C to kill cancer cells by direct cell necrosis, coagulation or carbonization (Nguyen and Kim 2016; Shah et al. 2015).

The challenge of this cancer therapy lies in controlling the heating effect to only the local tumour site so as to not harm the nearby healthy cells. To this end, magnetic hyperthermia has emerged as one of the most promising approaches for heat localization. Magnetic hyperthermia treatment is based upon the idea that magnetic nanoparticles heat up under an oscillating magnetic field. For this therapy, biocompatible magnetic nanoparticles are introduced into the tumour site either by direct injection or by targeted delivery. Oscillating magnetic field, or magnetic field gener-

ated by sending alternating current through a coil, is applied, and magnetic nanoparticles interact with this field to generate heat through various mechanisms.

The concept of magnetic materials in hyperthermia was first proven in 1957 when Gilchrist and co-workers heated various tissue samples with 20–100 nm size particles of γ-Fe_2O_3 exposed to a 1.2 MHz magnetic field (Demirci et al. 2018). Figure 10.12 shows a schematic representation of some of the unique advantages of magnetic nanomaterials for hyperthermia-based therapy and controlled drug delivery. Since, much progress have been made in the field using various types and sizes of magnetic materials, magnetic field strengths and frequencies, methods of preparation, coatings and nanoparticle delivery (Das et al. 2019; Abenojar et al. 2016; Hedayatnasab et al. 2017; Cruz et al. 2017). In 2007, Jordan and co-workers reported the first clinical study of magnetic hyperthermia, showing that aminosilane-coated superparamagnetic iron oxide nanoparticles could be safely applied for the treatment of brain tumours, achieving hyperthermic temperatures while being well tolerated by patients (Negut and Grumezescu 2019).

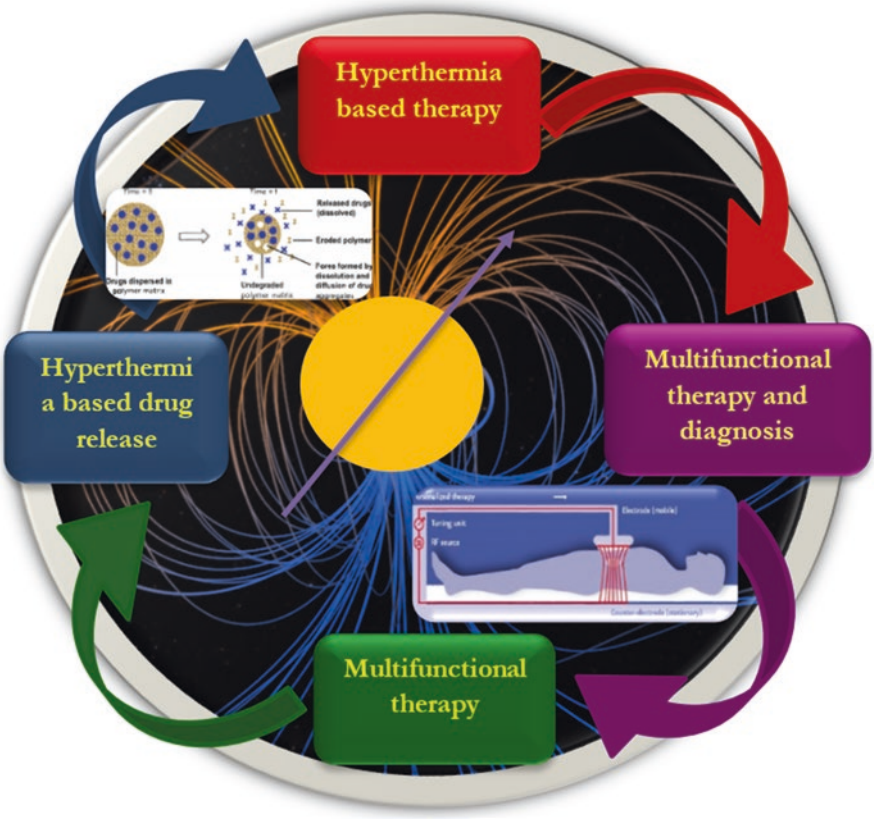

Fig. 10.12 A schematic representation of some of the unique advantages of magnetic nanomaterials for hyperthermia-based therapy and controlled drug delivery (Soica et al. 2018)

10.5 Conclusion

Diagnosis of diseases is very important in healthcare, which in turn not only enhances the effectiveness of medical treatment but is also effective in saving human life, where early diagnosis is crucial. However, in many cases early diagnosis needs sophisticated biomedical instruments or improved techniques. With regard to the unique properties of magnetic alloy nanoparticles (MANPs), its applications have expanded within the biomedical engineering creating a revolution in healthcare. The idealistic concept of a single platform for drug delivery to its monitoring of drug release seems to be feasible in the near future, because of recent advances in the application of novel nanomaterials in this field. The versatility of nanoparticles has been applied in various studies related to disease diagnostics, early detection studies and better contrast agents for improved imaging techniques. The development of new drug delivery vehicles has not only reduced the payload of the drugs but has also improved the efficacy of the drug in the system because of improved bio- and cyto-compatibility along with increased circulation time. Thus, the advent of nanoparticles has influenced all spheres pertaining to medical biotechnology and biomedical engineering, improving and enhancing the already existing techniques along with the experimentation of new and advanced techniques for drug delivery and its monitoring. In this article, two general fields of applications, namely, diagnosis (analytical biosensor/nucleotide interactions or visual bioimaging) and transportation (drug delivery and gene transfection), were discussed.

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Chapter 11
Hitching a Ride: Enhancing Nucleic Acid Delivery into Target Cells Through Nanoparticles

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Contents

Abstract Nucleic acids have gained significant interest in medicine for their therapeutic and prophylactic application. However, if delivered alone, nucleic acids are susceptible to nuclease degradation. Hence, delivering them with a suitable delivery system which can protect them could be beneficial. There is an increasing demand for novel delivery systems for nucleic acids to use them as vaccines and for gene

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therapy. Out of many types of delivery systems, nanoparticles are gaining importance because of their suitable properties. Hence, this chapter mainly focuses on discussing various types of nanoparticles for the delivery of nucleic acids. Recent applications of various types of nanoparticle-based viral and non-viral vectors and their advantages and disadvantages will be discussed in detail. The potential improvements which can be made to each existing nanoparticle systems are expressed. Overall this chapter is to provide an overview of importance of nanoparticles for nucleic acid delivery and is targeted towards beginners as well as advanced researchers in the field.

Keywords Nanoparticles · Vaccines · Gene therapy · Protective immunity · Nucleic acid delivery

11.1 Introduction

Nanoparticles are microscopic materials with a size range of 1–1000 nm, and the application of nanoparticles in pharmaceutical, food, drug, cosmetics and medicine has been increased over the past 20 years. An important medicinal application thereof has been in areas of vaccine and gene therapy. The amalgamation of two vast developing fields nanotechnology and vaccine delivery, mainly nucleic acid and antigen delivery, has led to development of a new cross-disciplinary field termed 'nanovaccinology' (Mamo and Poland 2012), which promises a prospective solution for prevention and therapy of many emerging diseases. Their application for delivering nucleic acids to the target cells is significantly increasing over the years, which can be stated based on the number of clinical trials. Nucleic acids are standing as an attractive option because of their ease of production and being economic. However, they need the aid of a safe delivery system to direct them to the target sites by preventing nuclease degradation. The number of nanoparticle-based nucleic acid delivery systems is discussed in detail in this chapter. Around 2600 clinical trials were completed for gene therapy by 2017 for various diseases including many types of cancers, Alzheimer's, diabetes, Parkinson's disease and immunodeficiency (Ginn et al. 2018). However only two, viz. type 1 Gaucher's disease and juvenile-onset hypophosphatasia gene therapy products, were approved for commercial use. This considerably lower success rate has been attributed largely due to the difficulty of delivering the nucleic acids to their target sites safely. Therefore, efficient delivery systems overcoming these barriers need to be developed to fill this gap. This book chapter provides an overview of current methodologies for nucleic acid delivery and further prospects in nanovaccinology.

11.2 Types of Therapeutic and Prophylactic Nucleic Acids

Human Genome Project and recent breakthrough researches elucidating the interconnected network of molecular pathways have facilitated a newer platform for the development of alternative therapeutic strategies with unprecedented target specificity. A majority of these strategies aim to strike at the core of the diseases by targeting the genes associated with the development and progression of it. The introduction of a new genetic material into the diseased cells with the intention of correcting or destroying the defective genes' expression responsible for the onset and progression of the disease is called 'gene therapy'. Nucleic acid-based therapeutics, a domain which initiated with the discovery of antisense technology, have presently included several other potent strategies like RNA interference (miRNA and siRNA), oligonucleotides (DNA and RNA aptamers, RNA decoy), catalytically active nucleic acids (DNAzymes and ribozymes), etc. Currently, researches are being conducted with these tools to yield promising drug candidates for treating a wide spectrum of diseases, including cancer, diabetes, infectious diseases and neurodegenerative and inflammatory diseases. Other than therapeutics, a rapidly evolving area of research is focusing on the development of prophylactic measures by using nucleic acids, for example, DNA and RNA vaccines. Here, we briefly review different nucleic acid-based therapeutics and prophylactics, their mode of action and their current status.

11.2.1 RNA Interference (RNAi)

RNAi is an evolutionarily conserved process in eukaryotic organisms by which short double-stranded non-coding RNA (ncRNA) triggers silencing of gene expression either by degradation of mRNA or by inhibiting its translation. In 1998, Fire, A. et al. discovered RNAi in the nematode worm *Caenorhabditis elegans* (Fire et al. 1998) and with another report published in 2001 by Elbashir et al. on silencing of genes in mammalian cell culture by short dsRNA molecules-small interfering RNA (siRNAs) (Elbashir et al. 2001), a new era of research on nucleic acid-based therapeutics has begun. Since its inception, RNAi has been greeted with overwhelming response in the biomedical community for its effectiveness as a tool to study the function of a gene and, thus, has ushered a fresh impetus for investigation of fundamental problems in biology at the level of gene expression. miRNAs, another class of endogenous small ncRNAs, was first discovered in 1993 as an important post-transcriptional regulator of gene expression (Lee et al. 1993; Wightman et al. 1993), both, siRNA and miRNA, remain at the fore front of biomedical research in RNAi technology. Both ncRNAs have similar properties but distinct functions which have been summarized in Table 11.1.

Table 11.1 Comparison of the properties of siRNA and miRNA (Carthew and Sontheimer 2009)

RNAi	siRNA	miRNA
Length	21–23 nucleotides with 2 nucleotides 3′ overhang	19–25 nucleotides with 2 nucleotides 3′ overhang
Complementarity to target mRNA	Fully complementary with very rare off-target exceptions	Full or partially complementary to target mRNA, preferentially targeting 3′ untranslated region of the target mRNA
Target	Regulate a single gene to which it is complementary or that expresses them	Regulate the same gene that expresses them and also the other genes to which they are partially complementary
Action	Cleave mRNA	Cleave target mRNA, inhibit gene expression
Application	Therapeutics	Therapeutic agent; diagnostic markers

Small Interfering RNA (siRNA)

The molecular mechanism of siRNA-mediated gene silencing is well established now (Lakatos et al. 2004). Following cellular uptake, long dsRNA is processed by an RNase III-like enzyme Dicer (endonuclease) in the cytoplasm into smaller siRNA fragments of 21–23 nucleotides (Bernstein et al. 2001). The siRNA interacts with RNA-induced silencing complex (RISC), a multiprotein complex, and activates it. A component of RISC complex, argonaute (AGO), then cleaves the passenger strand (sense strand) of the siRNA, resulting in its ejection from the assembly (Matranga et al. 2005; Rand et al. 2004, 2005). The guide strand (sense strand) remains associated with the RISC complex and guides it to the mRNA which contains the perfectly complementary sequence for binding leading to mRNA cleavage and gene silencing (Wittrup and Lieberman 2015).

A major setback in the early applications of siRNA as therapeutics was that the long dsRNA, once introduced in mammalian cells, readily elicited antiviral interferon response leading to cell death (Stark et al. 1998; Carthew and Sontheimer 2009). It has been observed that in vitro synthesis of siRNAs by T7 polymerase creates 5′-triphosphate which is responsible for triggering type 1 interferon response (Kim and Rossi 2007). Similarly, blunt-ended siRNAs trigger both cytoplasmic retinoic acid-inducible gene 1 protein (RIG1) and interferon production. Although many siRNAs are able to reduce target gene expression, they are also immunostimulatory in a sequence-independent manner because of their recognition by TLRs (Robbins et al. 2009). This immunostimulatory property has been smartly utilized in some studies of cancer immunotherapy. For example, CpG oligonucleotide (TLR9 agonist) and conjugated siRNA elicit an antitumor immune response in mice. However, this immune activation by siRNA is contraindicated in the other context (Kortylewski et al. 2009). Besides, aberrant immune activation, synthetically prepared siRNAs, once administered in the systemic circulation, are prone to get degraded by serum nucleases (Layzer et al. 2004; Jackson and Linsley 2010; Nguyen et al. 2012). In order to overcome these intrinsic limitations, a great length of arduous investigations was performed on the nature of chemical modifications of

siRNA structure without compromising its efficacy as well as on the delivery strategies of siRNA therapeutics.

To overcome aforementioned intrinsic limitations, investigations have been performed to chemically modify siRNA structure, in the sugar phosphate backbone, without compromising its efficacy and improving delivery strategies of siRNA therapeutics. Three types of chemical modifications in the sugar moiety have been extensively studied which include a 2′-O-methyl (2'-OMe), 2′-O-methoxymethyl (2′-OMOE) and 2′-fluoro (2′-F) modification in the sugar phosphate backbone (Deleavey et al. 2009; Judge et al. 2006). Either individually, or in combination with each other, these modifications are known to increase resistance to endonucleases. Both the 2′-OMe and 2′-F modifications bring A-form helical structure in the guide strand which in turn is suitable for positioning the target mRNA properly inside the RISC complex (Podbevsek et al. 2010). 2′-OMOE phosphorothioate at the 3′-overhangs of 21-nucleotide siRNA is perfectly complementary to pain-related cation channel P2X3 resulting in successful gene silencing (Dorn et al. 2004). A fully modified 2′-OMe and 2′-F guide strands are reported to have compromised activity. Therefore, alternating modifications of 2′-OMe and 2′-F have been studied and reported as appropriate for RISC loading and function (Allerson et al. 2005). Another modification, 2′-deoxy-2′-fluoro-β-D-arabino nucleic acid (2′-F-ANA), structurally similar to 2′-F has been shown to confer increased binding affinity to complementary mRNA and enhanced nuclease stability (Wilds and Damha 2000). 2′-F-ANA has applications in antisense technology also as it can mimic DNA structure that is able to trigger RNase H mediated RNA degradation (Souleimanian et al. 2012).

The 5′-phosphate stabilization is another kind of modification on siRNA that has been extensively studied. The 5′-phosphate at the guide strand of siRNA is essential for its binding to the effector protein AGO2 in the RISC complex. In order to preserve the 5′ phosphorylation, phosphonates can be used, for example, 5′-(*E*)-vinylphosphonate, most extensively studied phosphate analog. A recent report by Reka A. Haraszti et al. suggests that 5′-(*E*)-vinylphosphonate (5′-E-VP), a metabolically stable phosphate analog, confers better resistance to hydrophobically modified siRNAs not only from phosphatase but also from 5′-phosphate-dependent exonucleolytic destruction by XRN1, thus in turn enhancing overall potency, biodistribution, tissue accumulation and duration of activity (Prakash et al. 2015; Haraszti et al. 2017).

Delivery Strategy for Small Interfering RNA Therapeutics

In 2007, Quark Pharmaceuticals conducted the first clinical trial of systemic administration of siRNA. It involved the intravenous injection of naked siRNA (QPI-1002, also known as I5NP) designed to downregulate the expression of p53 in the kidney (Phase I and II trials; ID: NCT00802347) (Molitoris et al. 2009). Major drawbacks associated with delivery of naked siRNAs are different physiological challenges they have to face once they enter into systemic circulation, for example, i) stability

from serum nuclease, ii) evasion from immunosurveillance, iii) avoidance of non-specific interaction, iv) rapid renal clearance, v) target cell accumulation and loading into RNAi machinery, etc. (Yin et al. 2014). Therefore, a significant amount of researches has been conducted on developing better carriers for siRNA delivery. Lipid- and polymer-based carrier molecules have been extensively studied for facilitated delivery of siRNA, and more recently, with the progress of nanomaterial chemistry, several types of nanoparticle-mediated systemic infusion of siRNAs are undergoing clinical trials. Moreover, to achieve maximum benefits with least off-target effects, siRNA nanoformulations were conjugated with target specific ligand molecules. CALAA-O1 (Calando Pharmaceuticals; ID: NCT00689065) was the first targeted siRNA drug systemically administered via cyclodextrin polymer-based nanoparticle (Davis et al. 2010; Davis 2009). As the studies continued, several other conjugate systems like aptamers, peptides and antibodies have been used with different types of nanoparticle systems for increasing delivery efficacy.

MicroRNA (miRNA)

MicroRNAs, another class of short ncRNAs, function to regulate gene expression at the post-transcriptional level (Sharma et al. 2014). Initially, miRNA genes are transcribed by RNA polymerase II, resulting in the production of a dsRNA with a hairpin loop, called primary-miRNAs (pri-miRNAs) (Lee et al. 2003). These pri-miRNAs interact with the 'microprocessor' enzyme complex at the core of which resides an RNase III-like enzyme Drosha. Drosha cleaves the pri-miRNA to release a short hairpin structure called pre-miRNAs which are then transported into the cytoplasm from the nucleus via Exportin 5 complex (Lee et al. 2004; Lund et al. 2004). Like siRNA, pre-miRNAs then undergo Dicer-mediated processing leading to the formation of miRNA:miRNA∗ duplex (Hutvagner et al. 2001; Ketting et al. 2001). Subsequently, the miRNA∗ (passenger strand) is discarded while the mature miRNA (guide strand) is loaded into an RISC-like complex where AGO proteins bind it and direct it to bind to the target mRNA by complementary base pairing. On perfect binding or near perfect binding, target mRNAs are subject to cleavage and thus gene silencing occurs (He and Hannon 2004). Imperfect complementarity leads to the recruitment of other proteins by argonautes which ultimately brings translational repression. Thus, due to its ability to bring gene silencing even with partial complementarity with the target, a single miRNA can manipulate multiple gene expression successfully.

An improved understanding about the dysregulation of miRNA expression associated with a range of diseases leads to the development of oligonucleotides against those miRNAs anti-miRNA oligonucleotides (AMOs). Generally, AMOs hybridize with the target miRNAs and impair RISC loading. Chemical modifications have also been introduced in the structure of AMOs in order to enhance their stability, resistance from degradation by serum nuclease, hybridization affinity (Lennox and Behlke 2011). Krutzfeldt et al. reported that 3′ cholesterol-conjugated, 2′-OMe-modified oligonucleotides, known as 'antagomirs', sequestered miR-122 in the pri-

mate model of HCV infection and inhibited virus replication (Krutzfeldt et al. 2005). Miravirsen (SPC3649), currently undergoing a phase II clinical trial, is an LNA and PS modified 15-nucleotide sequence complementary to mature miR-122 a liver-specific miRNA that the hepatitis C virus (HCV) requires for replication (Gebert et al. 2014).

The function and maturation of pro-tumorigenic cluster of miRNAs have been shown to attenuate by some aptamers (detailed elsewhere). Subramanian N. et al. reported an RNA aptamer (aptamer 7) which was shown to abrogate the maturation of oncogenic miRNAs, miR-17, miR-18a and miR-19b and thus able to induce apoptosis in retinoblastoma cells in vitro (Subramanian et al. 2015a).

11.2.2 Nucleotides

An increasingly deeper insight about the contribution of aberrant expression of genes to the development and progression of a wide spectrum of inherited and acquired diseases constitutes a new domain of therapeutic strategies based on altering the expression of particular genes. Besides antisense technology, several nucleotides with the ability of gene activation and suppression have been developed, for example, decoy oligonucleotides, aptamers, etc.

Antisense Oligonucleotides

Antisense oligonucleotides (AONs) work by interfering with the mRNA translational machinery, either by sterically hindering ribosome binding to mRNA sequence or by degrading the target mRNA via the enzyme RNase H (Jain et al. 2012). Fomivirsen (brand name Vitravene), a phosphorothioate oligonucleotide, was the first FDA-approved AON antiviral drug, used for the treatment of cytomegalovirus (CMV) infection (Mulamba et al. 1998).

To be successful as effective therapeutic agents, AONs must possess high affinity and target specificity to displace any RNA secondary structure or competitively prevent the binding of transcription factors to the target RNA. On the other hand, cleaved RNA fragments must be released from their bound AON so that it can further bind and cleave the next target, so excessive high binding affinity can lead to a reduced potency (Pedersen et al. 2014). To overcome the inherent limitations of the poor binding affinity of naturally occurring oligonucleotides and low degree of nuclease resistance, researchers have been focusing on the introduction of chemical modifications of different moieties of oligonucleotides (Fig. 11.1) (Micklefield 2001; Corey 2007). Consequently, depending on these chemical modifications the AONs have been classified into three generations which are summarized in Table 11.2. A significant amount of research has focused on the development of various in vitro and in vivo deliveries of AONs.

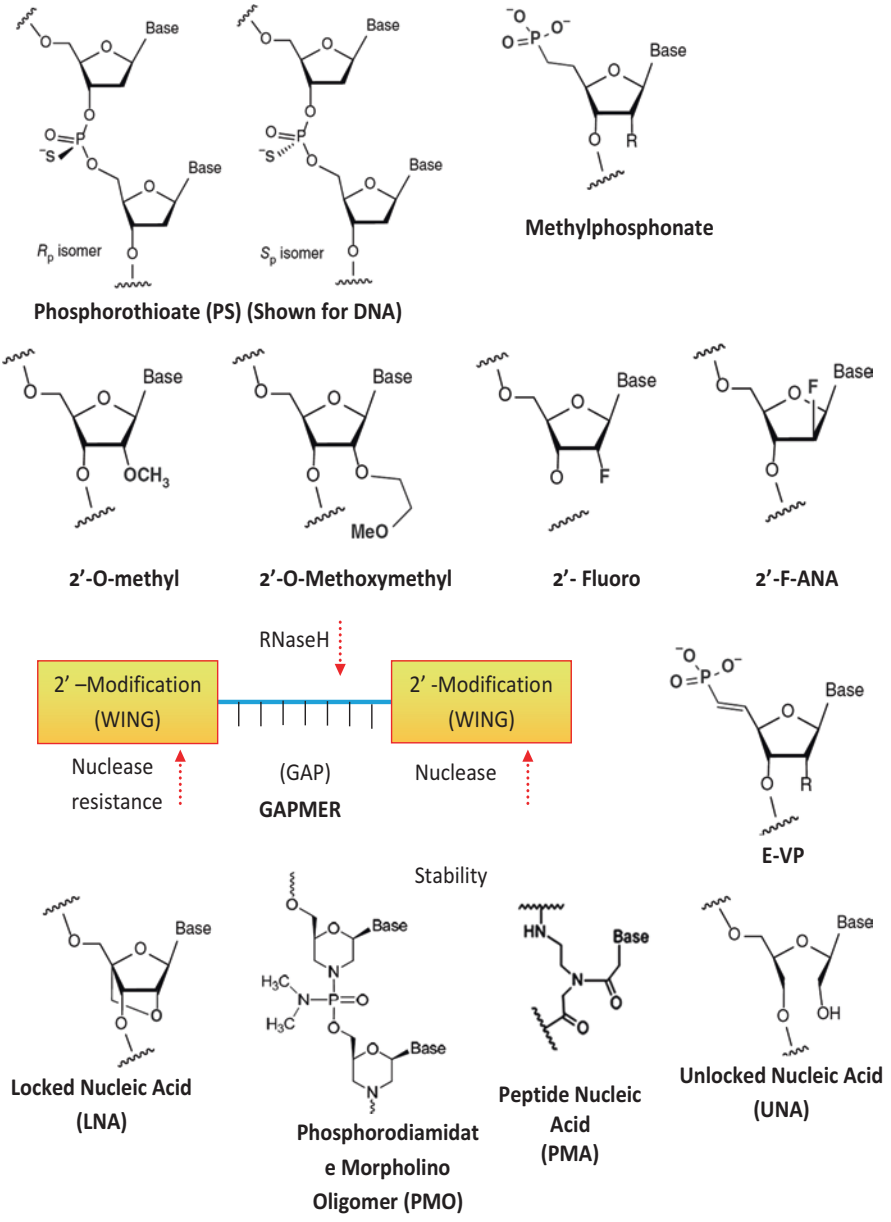


Fig. 11.1 Structure of chemical modifications of different nucleotides used for antisense therapy. Combinations of these modifications yield highly potent therapeutic drugs

Table 11.2 Different chemical modifications and generations of antisense oligonucleotides

Generation	Examples of modification	Characters
First generation example: 'Fomivirsen' (brand name 'Vitravene') (Mulamba et al. 1998)	Phosphorothioate (Xie et al. 2012; Rahman et al. 2012), Methylphosphonate (Rahman et al. 2012; Shoji et al. 1991)	RNase H-dependent activity (Agrawal and Kandimalla 2000) Generally prepared as diastereomeric mixtures (Agrawal and Kandimalla 2000) 'S'-diastereomer: Higher stability, more resistance to nuclease than natural oligonucleotides (Eckstein 2002) 'R'-diastereomer: Improved binding affinity than natural ONs (Eckstein 2002) Easier delivery inside the target cells due to negative charges Suitable pharmacokinetic properties (Agrawal and Kandimalla 2000) Delayed renal clearance Require comparatively higher doses
Second generation example: 'Kynamro' (McGowan et al. 2012)	2′-O-methyl (Deleavey et al. 2009; Prakash 2011), 2′-O-methoxyethyl (Prakash 2011), 2′-fluoro (Deleavey et al. 2009), 'GAPMER' (Pedersen et al. 2014; Seth et al. 2012)	Augmented binding affinity towards complementary RNA Improved nuclease resistance, half-life and hybridization stability Comparatively lower doses are required A substantial lowering of immune stimulation Incapable of induction of 'RNase H' activity except for the chimeric AON, 'GAPMER' (a central 'gap' of deoxyribonucleotides, hemmed between terminal blocks of 2′-O-modified ribonucleotide 'wings' (Pedersen et al. 2014; Seth et al. 2012)

(continued)

Table 11.2 (continued)

Generation	Examples of modification	Characters
Third generation PMO class of drug—Exondys 51 (eteplirsen, made by Sarepta therapeutics, used for the treatment of Duchenne muscular dystrophy)	Locked nucleic acid (LNA), phosphorodiamidate morpholino oligomer (PMO), peptide nucleic acid (PNA) (Kurreck 2003; Gleave and Monia 2005)	A. Locked nucleic acid (LNA) 1. Ribose sugar ring is constrained in the 3′-endo conformation by a methylene bridge between 2′-O and 4′-C (Prakash et al. 2008; Hildebrandt-Eriksen et al. 2012) 2. Unprecedented thermal stability and binding affinity (Veedu and Wengel 2010) 3. Being unable to activate RNase H, LNA monomers are conjugated with RNA or DNA to form chimeric 'gapmer' which exhibits efficient mRNA cleavage (Veedu and Wengel 2010)
		B. Phosphorodiamidate Morpholino oligomer (PMO) 1. Steric blocker; cannot activate RNase H (Amantana and Iversen 2005) 2. Ribose sugar is replaced by a six-membered morpholino ring, and the phosphodiester bond is replaced by a phosphorodiamidate-linkage (Schnell et al. 2013) 3. Better nuclease resistance 4. Target affinity is the same to that of the natural AON identifying the similar sequence (Amantana and Iversen 2005) 5. Uncharged, cellular uptake is rather difficult (Amantana and Iversen 2005) 6. Reduced non-specific interaction
		C. Peptide nucleic acid (PNA) 1. Nucleobases attached to a pseudopeptide polymer N-(2-amino-ethyl) glycine backbone by a methylenecarbonyl linkage (Banerjee and Kumar 2013) 2. Steric blocker; cannot activate RNase H (Ji and Lei 2013) 3. Mostly uncharged (Ji and Lei 2013) 4. Strong binding affinity

Decoy Oligonucleotides

With the growing interests in the genetic basis of disease pathogenesis, transcription factors and several other molecular regulators are increasingly being regarded as suitable therapeutic targets. Each transcription factor binds to a cognate sequence of 6–10 base pairs in length (single or multiple copies) at the promoter region of the

target genes whose expression is regulated (Sharma et al. 2014). As this binding is a highly selective phenomenon, synthetically prepared short stretches of oligonucleotides, named as decoy oligonucleotides (dODNs) carrying transcription factor-specific consensus binding sites, can competitively bind the target sequence, once delivered inside the cell and rendering the transcription factors incapable of subsequent binding to the promoter regions of the target genes. This, in turn, can stop the transcription factor from regulating the expression of its target gene (Yuan et al. 2013; Ahmad et al. 2013).

A wide variety of chemical modifications on dODNs has been tested to increase their efficacy. Zhang Q et al. demonstrated that the dODNs against STAT3 were specifically delivered to myeloid cells by conjugating with the TLR9 ligands, cytosine guanine dinucleotide (CpG). Phosphorothioate modifications were also brought partially within the CpG arm and STAT3dODNs arm (Zhang et al. 2016). Double-stranded dODNs with phosphorothioate modifications were also reported to bind specifically transcription factor NF-kβ (nuclear factor kappa-light-chain-enhancer of activated B cells). These phosphorothioate-stabilized ODNs show enhanced serum stability (Lesage et al. 2003). In order to increase stability, dODNs were also developed on PNA-DNA chimeric chemistry which is more resistant to exonuclease than DNA-DNA hybrids. Borgatti M et al. reported that the dODNs based on a PNA-DNA-PNA (PDP) chimera possessing the Sp1 binding sites effectively impair Sp1-DNA interaction (Borgatti et al. 2003). Along with stability, another major area of emphasis is to design delivery strategies for increasing target specificity, cellular uptake and minimizing off-target effects of dODNs. Therefore, cationic liposomes and biodegradable microspheres have been tested (Ahmad et al. 2013; Ungaro et al. 2012).

Aptamers

Aptamers are small single stranded DNA or RNA oligonucleotides (ONs) capable of binding with high affinity and specificity to target molecules, ranging from small inorganic molecules to large protein complexes or an entire cell (Sharma et al. 2014; Ellington and Szostak 1990; Tuerk and Gold 1990). Pegaptanib sodium injection (brand name Macugen), an antagonist to vascular endothelial growth factor (VEGF), is the first FDA-approved aptamer-based drug (Ng et al. 2006). The advantages of aptamer-based therapeutics over other ON-based strategies are their smaller sizes, non-immunogenicity and cheaper production cost (Ferreira and Missailidis 2007).

The most conventional way of aptamer production is known as SELEX (systematic evolution of ligands by exponential enrichment). A large library of 10^{14} to 10^{15} different random DNA or RNA oligonucleotide sequences is incubated with the target molecule. DNA or RNA molecules which bind to the target ligands are separated from those which do not bind. The bound oligonucleotides are PCR amplified and undergo further selection. Thus, the enrichment process is continued until oligonucleotides with high affinity to the target molecules can be isolated and cloned (Avci-Adali et al. 2013; Dwivedi et al. 2013; Bouchard et al. 2010). CELL-SELEX,

a relatively newer cell-based SELEX method, provides aptamers against the whole cell. This method has several advantages over the traditional method. For example, CELL-SELEX overcomes the limitation of purified recombinant proteins as required in the traditional method (Catuogno and Esposito 2017). As in this method, all the cell surface molecules are in their native state, and the generated aptamers will bind to the real folded conformation. Therefore, CELL-SELEX also significantly helps in biomarker discovery.

Aptamer-based therapy also has some limitations, for example, degradation by serum nuclease, rapid renal clearance, etc. Like other oligonucleotides, in order to increase the resistance against nuclease, use of chemically modified bases is preferred in aptamer-based therapy. For example, Macugen includes 2′-fluoronucleotide (2′-F) and 2′-O-methyl (2′-OME) nucleotides and is conjugated with a 40-kDa polyethylene glycol (PEG) to avoid rapid renal clearance with increased half-life of the systemic circulation (Ng et al. 2006). Another approach to enhance the stability of aptamers is to develop 'mirror aptamers' or 'spiegelmers'. Spiegelmers have an oligonucleotide backbone comprising entirely of **L**-ribose (RNA spiegelmers) or **L**-deoxyribose (DNA spiegelmers). Serum nucleases, being highly stereoselective, are not able to degrade L-nucleotides (Hoffmann et al. 2011). The advantages of aptamer-based strategies over the conventional AMOs-based approaches are that RNA aptamers can bind to different sites within a miRNAs cluster and thus can regulate multiple miRNAs, whereas commonly used AMOs owing to binding with target mRNA via direct complementarity can target only single miRNAs (Subramanian et al. 2015a).

Currently, a major focus is being provided to develop aptamers as 'escort' signals to deliver drugs or other cargo molecules to their target sites (Hicke and Stephens 2000). For example, A10 RNA aptamers that bind to the prostate-specific membrane antigen (PSMA) protein on the prostate cancer cell membrane target specifically transport doxorubicin (Dox) to the cancer cells (Bagalkot et al. 2006). Successful delivery of siRNAs to their target sites is also often mediated by conjugating the siRNAs with aptamers. One of the first studies reported the development of siRNA coupled with anti-PSMA aptamer (A9) via biotin-streptavidin bridge by Chu et al. This siRNA-aptamer conjugate demonstrated comparable gene knockdown efficiency with the conventional lipid-based delivery reagent (Chu et al. 2006). Zhou et al. designed a cell type-specific delivery strategy of anti-human immunodeficiency virus (anti-HIV) siRNA (tat/rev) by conjugating the siRNA with anti-gp120 aptamer where the aptamer targets the cells expressing gp120, an HIV-specific glycoprotein, facilitating viral entry into host cells. Gene silencing efficacy by siRNAs strictly depends on their sufficient release to the intracellular target sites (Zhou et al. 2008). In order to achieve satisfactory release from their carrier, instead of direct conjugating siRNAs with aptamers, often they are loaded into functional polymers or nanoparticles which on the other hand are guided by aptamers as targeting ligands. This ensures better siRNAs accumulation at the target sites as nanoparticles are easily internalized by adhering target cells (Subramanian et al. 2015b; Zhou and Rossi 2014). Aptamer-nanoparticle complexes have also been useful in other clinical purposes, for example, designing biosensors, and target specific diag-

nostic imaging and therapy. Medley et al. developed a simple and rapid colorimetry-based detection protocol of cancer cells by using aptamer-conjugated gold nanoparticles (AuNPs), where aptamer-AuNP complexes were shown to bind the target cells and thus interparticle distance between AuNP decreased which in turn produced visible colour changes from red to violet, resulting in straightforward detection of cancer cells (Medley et al. 2008). Aptamer-modified dye-doped silica nanoparticles (SiNPs) are effectively utilized as valuable diagnostic probes. Jo H et al. developed a dual aptamer-based detection platform for cancer cells where SiNPs were conjugated with an MUC1 aptamer and a human epidermal growth factor receptor 2 aptamer, thus making the SiNPs highly selective for only the MUC1(+) and HER2(+) cell lines (Jo et al. 2015). Like siRNA, several groups recently explored the aptamer-guided delivery of 'tumour suppressor' as well as other beneficial miRNAs. Liu et al. combined anti-MUC1 aptamer to let-7i miRNA and thus sensitized OVCAR-3 ovarian cancer cells to paclitaxel (Liu et al. 2012). Carla L Esposito et al. developed another multifunctional aptamer-miRNA conjugate by combining a tumor suppressor Let-7 g miRNA to anti-Axl receptor inhibitory aptamer named 'GL21.T'. Importantly, this study clearly demonstrated reduction of tumor growth in tumor xenograft model of lung adenocarcinoma (Esposito et al. 2014). We already have discussed how aptamers have also been therapeutically used to impair the function of oncogenic miRNAs. Thus aptamers provide a wide array of opportunity of gene delivery, making it an emerging area of research.

11.2.3 Catalytic Nucleic Acids

The last few decades have seen a significant expansion of research interests on the catalytic nucleic acids. As a non-protein machinery equipped with the ability to manipulate biomolecules, catalytic nucleic acids have been extensively studied which has established their credential as an important therapeutic tool. Two types of catalytic nucleic acids, for example, ribozymes and DNAzymes, have received major attention as therapeutically important devices.

Ribozymes

Ribozymes are catalytic RNA that can act as an enzyme without having any protein like properties (Kiehntopf et al. 1995). Most natural ribozymes are indigenously capable of catalysing target mRNA cleavage and ligation. Among a vast array of ribozymes, mainly two types, viz. hammerhead and hairpin ribozymes, have gained therapeutic importance due to their small size and highly efficient cleavage reaction (Haseloff and Gerlach 1988; Fedor 2000; Rossi et al. 1992). In order to enhance the therapeutic efficacy and protect RNases, several chemical modifications in ribozymes' structure have been studied which include incorporation of 2′-O-methyl group on sugar moiety of RNA monomers, 2′-deoxy-2′-*C*-allyl uridine, phosphoro-

thioate linkage (Heidenreich et al. 1994; Burnett and Rossi 2012; Beigelman et al. 1995) and terminally inverted 3′-3′ deoxy-sugar. Substrate recognition domain of ribozymes is also often artificially engineered to induce site-specific cleavage. The first synthetic ribozyme that underwent clinical trial was angiozyme which targets the mRNA of the vascular endothelial growth factor receptor-1 (VEGFR-1). Several ribozymes have been subjected for clinical trials to treat viral infection and cancer (Usman and Blatt 2000).

Deoxyribonucleotide Enzymes (DNAzymes)

Deoxyribonucleotide enzymes or DNAzymes are small DNA molecules capable of catalysing various types of reactions. Most of the DNAzymes facilitate mRNA cleavage. So far, DNAzymes have not been isolated naturally. They are generally produced by a combinatorial biology technique called in vitro selection (Sun et al. 2000). Till date, the most extensively studied DNAzyme is 10–23 DNAzyme (its origin as the 23rd clone of the tenth cycle of in vitro selection) which has shown extraordinary ability to cleave mRNA sequence with high specificity provided it contains a purine-pyrimidine dinucleotide (Santoro and Joyce 1998). Their catalytic activity mostly depends on the metal ion cofactor. One big advantage of the therapeutic use of DNAzymes over antisense oligonucleotide technology is that it can bind and slice the target mRNA without requiring RNase H. DNAzymes have also been used as biosensors. For example, Torabi SF et al. reported a highly specific Na^+-dependent DNAzyme named NaA43 which is significantly active at a physiological concentration of Na^+ ion and, hence, can be used as a biosensor for intracellular detection Na^+ ions (Torabi et al. 2015). Till date, DNAzymes have shown its therapeutic credential against various diseases. Very recently, M. Chakravarthy et al. published another DNAzyme candidate RNV143 targeting ITG4 transcript as a potential therapeutic strategy to reduce inflammation in multiple sclerosis (Chakravarthy et al. 2017).

11.2.4 Nucleic Acid Vaccines

Nucleic acid-based vaccines are one of the boons of recombinant DNA technology which has triggered a shift of paradigm in age-old vaccination strategy. In this process a gene fragment encoding the antigens of interest is delivered into host cells and directly expresses antigen protein in situ. Nucleic acid vaccines have already received significant attention due to several reasons, most important of which is that the immune response to the introduced vaccines can either be directed to stimulate either of the humoral or cellular immune response or both without the need live vectors. Thus the potential risk of unwanted acute infection in conventional first- and second-generation vaccination strategies, sourced from live-attenuated or killed pathogens, polysaccharides, proteins or synthetic peptides, can be avoided (Hasson

et al. 2015). Moreover, in comparison to recombinant protein antigen they are much easier to synthesize and hence cheaper. Nucleic acid vaccines are of two types, 1) DNA vaccines and 2) RNA vaccines. Here we will briefly review about these two types of vaccines and highlight the present status of their application.

DNA Vaccines

DNA vaccines are bacterial plasmids encoding antigenic protein following in vivo administration and subsequent transfer into the cells (Liu 2011). The extent of immune response via DNA vaccine depends on i) the mode and site of gene delivery, (ii) the plasmid dose and (iii) the administration of booster injections. In general, DNA immunization is done in two ways, i) intramuscular or intradermal injection and ii) gene gun delivery (Hasson et al. 2015; Raz et al. 1994; Diniz and Ferreira 2011). The incorporated DNA vaccine is expressed in the injected muscle cell and in surrounding APCs. The proteins are being processed as endogenous antigens through the MHC class I pathway, and peptides are expressed on the surface of both cell types. MHC class I cells stimulate cytotoxic T lymphocytes. The protein encoded by the injected DNA may also be expressed as a soluble protein which is taken up, processed and presented through class II MHC molecules. This pathway triggers B-cell immunity and produces antibodies. Thus, the DNA vaccines can elicit both the humoral and cellular immunity (Hasson et al. 2015; Ferraro et al. 2011). In last few decades, DNA vaccines have been evaluated for several diseases, majorly for HIV and cancer. For example, human papillomaviruses (HPV) cause cervical carcinoma, and viral E6 and E7 oncoproteins are suitable vaccine targets. A DNA vaccine against HPV type 16 has been reported (Yan et al. 2009).

RNA Vaccines

Although, initially, plasmid-based DNA vaccines gained major research interests, messenger RNAs were also investigated as potentially safer alternative of DNA vaccines. The major advantage of RNA vaccine over the DNA vaccine is that unlike DNA vaccine, there is no risk of insertional mutagenesis due to vector integration into the host genome and consequent generation of malignancies for RNA vaccine. Secondly, owing to shorter half-life, expression of RNA occurs transiently due to which host exposure to the antigen is very controlled which, in turn, minimizes the risk of tolerance induction (Johansson et al. 2012; Pollard et al. 2013). Certain RNA molecules ignite strong immune response resulting in induction of an efficient adaptative immune response. For example, double-stranded RNA molecules, normally absent in mammalian cells but produced during viral replication, are recognized by host cell as a signal to cast strong immune activation (Jin et al. 2010). During vaccination, RNA can be administered naked, in liposomes, or coated onto the nanoparticles. Recently, Romani B et al. encapsulated RNA transcripts with DOTMA and DOPE lipids and transfected into

mice. The report suggests that the RNA vaccine showed generation of humoral immunity against and recover mice from influenza A virus infection (Romani et al. 2017). RNA vaccines already have shown to play a promising role in cancer immunotherapy. Kuhn AN et al. showed that phosphorothioate (D1 stereoisomer)-modified end capping ($m_2^{7,2'-O}$ Gpp_SpG (β-S-ARCA)) increases stability and translation efficiency of RNA vaccines in immature dendritic cells and thus can trigger efficient induction and proliferation of naïve antigen-specific T cells in mice (Kuhn et al. 2010). There are no specific treatments or effective vaccines available against Zika virus. mRNA-based vaccines have been proved to be an effective platform for treatment. A lipid nanoparticle containing mRNA with the modified nucleoside 1-methylpseudouridine (m1Ψ) encoding the pre-membrane and envelope glycoproteins of a zika virus strain showed protection against zika virus challenges in mice at 2 weeks or 5 months after vaccination (Pardi et al. 2017).

A new class of RNA molecules, known as self-amplifying mRNA (RNA replicons), has emerged as a promising vaccine candidate which is derived from either positive-strand or negative-strand RNA viruses with the viral structural genes replaced by genes encoding antigen of interest. Self-amplifying RNA vaccines have been successfully employed with different antigens in several animal species, including mice, nonhuman primates and humans (Rodriguez-Gascon et al. 2014).

11.3 Mechanism of Action of Nucleic Acid Vaccines

Nucleic acid vaccines including DNA- and RNA-based vaccines have the similar mechanism of action with the ultimate aim to elicit antigen-specific immune responses. The nucleic acids can be delivered to somatic cell or germ cell based on their application. For example, DNA vaccines for cancer can be delivered to the target somatic tissue, whereas the nucleic acids encoding protein to prevent inherited chromosomal disorders can be introduced into germ cells. However germ cell gene therapy is not well developed due to the ethical and technical issues (Verma et al. 2000). In general description, nucleic acid vaccines utilize the host transcriptional and translational machinery to encode protein which can act as antigen.

Plasmid DNA encoding an immunogenic part of a pathogen can be introduced into somatic tissue or directly into antigen presenting cells depending on the targeted disease (Fig. 11.2) (Xu et al. 2014). The antigens encoded by DNA vaccines can directly activate B cells resulting in antibody production, and antigen presenting cells (APCs) can be activated after cross-presentation (indirect transfer) of antigens. Thus, DNA vaccines have the capacity to elicit both arms of the immune response, which is beneficial.

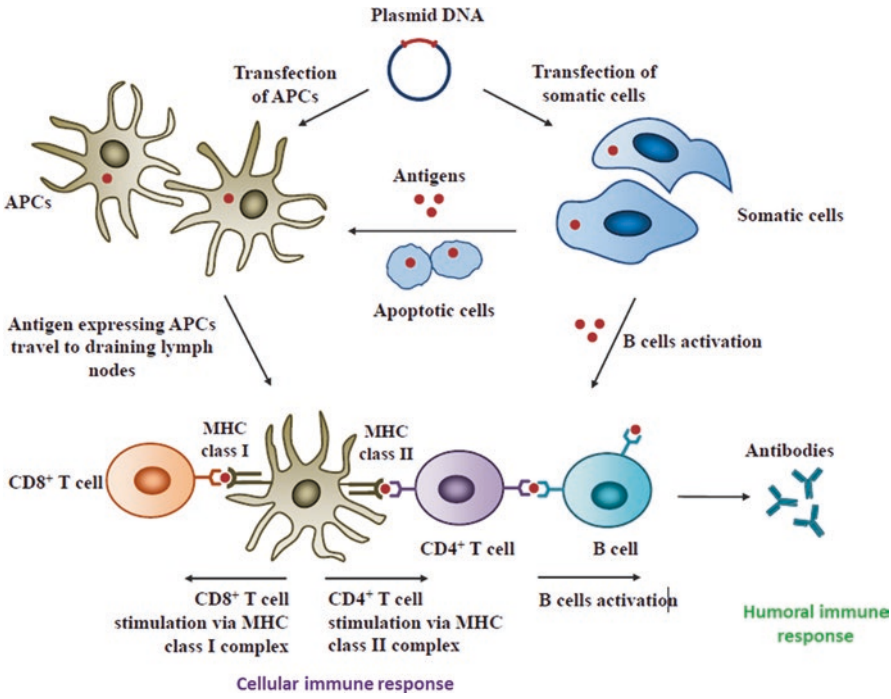


Fig. 11.2 Mechanism of action of nucleic acid vaccines. (Modified from reference (Xu et al. 2014))

11.4 General Methods of Nucleic Acid Delivery

The first-generation nucleic acid delivery was achieved by the direct administration of nucleic acids into the system. Even though the direct injection of nucleic acids has got a reasonable success in vitro, they are not preferred to be used alone. The reasons may be due to their large size and shape which makes it difficult to enter the pores on the cells easily and their negative charge makes them less able to be uptaken by negatively charged cell membrane (Al-Dosari and Gao 2009). Naked DNA vaccines can induce inflammatory Th response but require multiple booster dosages to elicit effective immune responses. They are also susceptible to digestion by serum nucleases. To overcome these disadvantages of naked DNA vaccines, many research groups dedicated to developing novel delivery systems. The second- generation of DNA delivery was favoured by various physical methods such as gene gun and electroporation. The third-generation nucleic acid vaccine delivery included employing various delivery vehicles synthesized by biological and chemical means. Each method is summarized in Fig. 11.3.

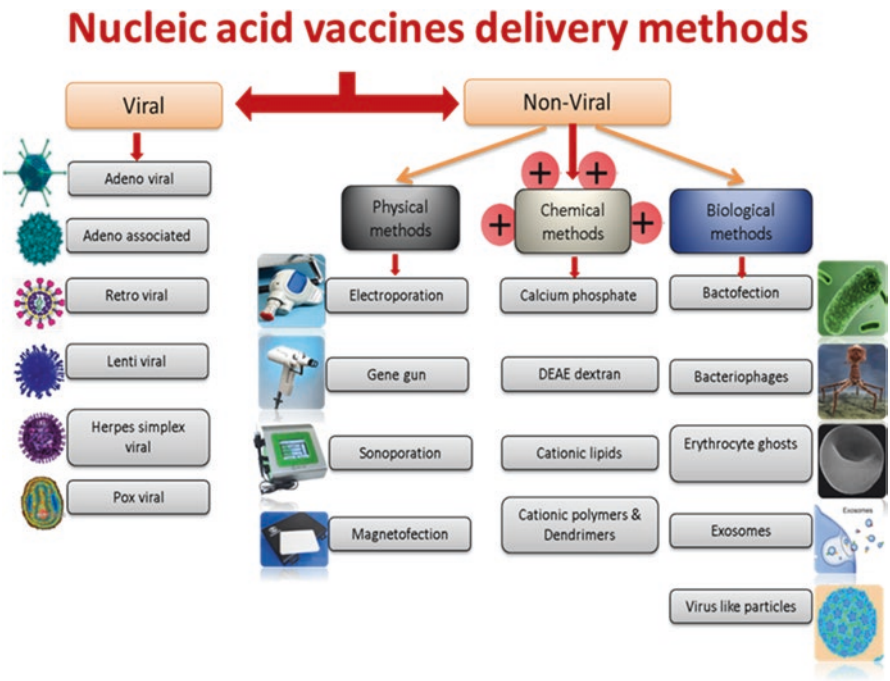


Fig. 11.3 General methods of nucleic acid delivery

Traditional methods of nucleic acid vaccine delivery mainly involve direct injection of naked nucleic acid into tissue, and in most cases, this results in very low transfection efficiency (Barry et al. 1999). A recent study indicated that the half-life of naked nucleic acid in body fluids was relatively lower compared to that of a nucleic acid complexed with protein (Yao et al. 2016). This can be supported by the fact that cellular DNA is associated with histones and hence protected by nuclease degradation. To overcome this, various delivery systems were developed.

11.5 Challenges in Nucleic Acid Delivery

The pathway of nucleic acid delivery encounters various barriers which can be further divided into extracellular and intracellular barriers. Each barrier is summarized in Fig. 11.4 and is discussed below.

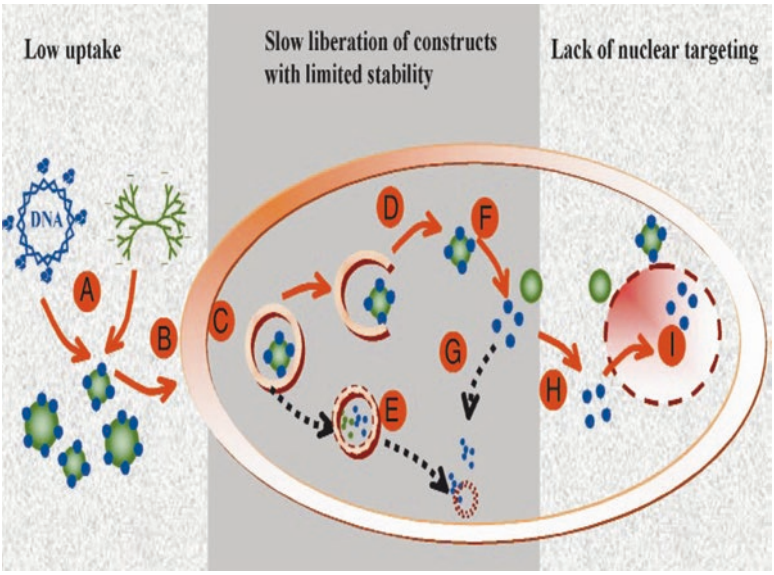


Fig. 11.4 Schematic representation of DNA delivery pathway pointing three major barriers. (**a**) DNA-complex formation. (**b**) Uptake. (**c**) Endocytosis (endosome). (**d**) Escape from endosome. (**e**) Degradation (within endosome). (**f**) Intracellular release. (**g**) Degradation (within cytosol). (**h**) Nuclear targeting. (**i**) Nuclear entry and expression. (Figure adapted from (Luo and Saltzman 2000))

11.5.1 Extracellular Barriers

The foremost obstacles a nucleic acid encounters before entering the cell include difficulty in uptaking by the plasma membrane. The nucleic acids, being an polyanionic macromolecule, cannot passively diffuse across the plasma membrane and require transport mechanisms such as ion gradient diffusion (Grinius 1980) and endocytosis. For instance, the uptake of plasmid DNA can be affected by various factors such as size of plasmid DNA, charge of complexes and protection of DNA from extracellular nucleases (Jorritsma et al. 2016). The cell being negatively charged preferentially uptakes cationic material compared to that of anionic substances. Thus, the naked DNA needs to battle with the cell membrane to enter the cell which can make a long halt for nucleic acid outside the cell making it more susceptible to extra cellular nucleases. Hence, it will be beneficial to complex nucleic acids with a material which can protect it. Similar consequences can be observed with the larger size of nucleic acids and require a material to condense them to enter the cell easily.

11.5.2 Intracellular Barriers

The nucleic acids, after successful entry into the cell by endocytosis, require passing through the endosomal pathway before it enters the nucleus for transcription. The late endosomes contain acidic pH with acid hydrolases which cleave the encapsulated material before releasing them into the cytoplasm. The naked nucleic acid can be degraded at this step, if not protected. Hence, the nucleic acid should ideally escape the endosome at the early stages to reach the nucleus. Nucleic acid complexed with cationic polymers and cationic lipids have been reported to escape from the endosomes by preventing endosomal acidification and membrane destabilisation (Nguyen and Szoka 2012). Endosomal pathway is very important for non-viral nucleic acid delivery, and most of the non-viral vectors are designed to aim the early endosomal escape (Luo and Saltzman 2000).

11.6 Vectors for Nucleic Acid-Based Vaccines

The economic convenience of nucleic acid-based vaccines compared to recombinant protein-based vaccines resulted in the invention of various types of nucleic acid-based delivery methods. Second-generation DNA delivery was favoured by various methods such as gene gun and electroporation. The third-generation DNA vaccine delivery included employing various delivery vehicles synthesized by biological and chemical means. Each method is discussed in detail in the following sections.

11.6.1 Virus-Based Delivery Systems

Viral-based nucleic acid delivery is one of the familiar methods used against many diseases (Nayerossadat et al. 2012). The first application of virus for gene delivery can be dated back to 1972 when a recombinant DNA was synthesized using SV40 virus (Jackson et al. 1972), and by the successful application of the vaccinia virus for transient gene expression (Mackett et al. 1982). The basic working principle behind utilizing viruses for nucleic acid delivery is based on their capability to introduce its own genetic material into the host cell during the infection phase of life cycle. The nucleic acid vaccines may encode an immunogenic or therapeutic protein- encoding gene cassette, which will be packed or coated on to the virus, ready to lodge it into the host cell (Kay et al. 2001). Only a few viruses are suitable for this kind of application and must be selected based on their properties. Despite having high transfection efficiency, their use is limited due to insertional mutagenesis making them unsafe and often immunogenic (Kim and Eberwine 2010). However, each type of viral system has its unique advantages and limitations. The most common

types of viruses used for vaccine delivery and their general advantages and disadvantages of each system are summarized in Table 11.3.

Adenoviral Vectors

Background

Adenovirus is a linear non-enveloped icosahedral virus, sized 70–100 nm, having double-stranded DNA as the genetic material (Crystal 2014). This virus was first isolated from cell cultures developed from adenoid tissue in the 1950s and was named based on its source of isolation. These viruses gave rise to first-generation replication-incompetent vector, followed by second-generation replication competent vectors, forming the basis for most of the preclinical development and clinical trials (Appaiahgari and Vrati 2015). The high-capacity adenoviral vectors constitute the third-generation adenoviral vectors and are also termed as gutless adenoviral vectors, as these are stripped off of all the viral coding sequences. These vectors are only characterized with 5′ and 3′ inverted terminal repeat sequences, allowing for accommodation of up to 36 kb of transgenic cloning sequences (Lee et al. 2017). The adenovirus (Ad) vectors possess the ability to transduce both replicating and

Table 11.3 Major types of viral systems available for gene and plasmid DNA delivery, their merits and demerits

Transfection systems	Merits	Demerits
Adenoviruses vectors	Large transgene capacity (up to 38 kb), low host specificity	Tend to yield natural and acute immunologic responses, short-term gene expression
Adeno-associated vectors	Safety, ability to integrate into a specific site on chromosome 19 with no noticeable effects	Complicated process of vector production and the limited transgene capacity (up to 4.8 kb)
Retroviral vectors	Ability to transfect dividing cells, suitable for in situ treatment, transgene capacity of 8 kb	Low efficiency in vivo, immunogenic problems, the inability to transfect the non-dividing cells and the risk of insertion
Lentivirus vectors	High-efficiency infection of dividing and non-dividing cells, long-term stable expression, low immunogenicity, transgene capacity of 8 kb	Difficult design and construction, concerns of biosafety
Herpes simplex virus vectors	Transgene capacity of up to 150 kb, neuronotropic features	Difficulty to keep virus action under control
Poxvirus vectors	High stable insertion capacity (more than 25 KB), simple construction, high expression levels	Complex structure and biology, risk of cytotoxic effects

Table adapted with minor modifications from (Jin et al. 2014)

quiescent cells, exhibit high immunogenicity and can trigger antitumor immunity. Adenoviral DNA does not integrate into the genome of the host and rather exists as an episome (Vannucci et al. 2013).

Application for Nucleic Acid Delivery

Ad vectors find application in different types of therapies such as suicide gene therapy, immunotherapy, gene replacement techniques and in combination with chemotherapy.

Anticancer Agents Ad vectors form three therapeutic categories with respect to anticancer treatment. The first category involves replication-defective vectors which deliver the immune-related genes directly to tumorous cells. The second category involves the introduction of replication competent vectors in the cancerous cells. They execute their natural lytic life cycle, resulting in oncolysis of cancer cells. The third category includes the delivery of tumor suppressor or cytotoxic genes, using either replication defective or competent to induce cytotoxic cascade resulting in cell cycle arrest or apoptosis.

Vaccine Development Ad vectors have been utilized in preparing vaccines against tuberculosis, malaria, HIV, influenza and Ebola. These vaccines are known to exhibit a strong T-cell and humoral response. The responses generated from these vaccines are more robust and highly efficient in destroying infected cells, cancerous cells and pathogens.

Advantages and Disadvantages

The advantages and limitations of Ad vectors in nucleic acid delivery have been summarized in Table 11.4.

Improvements that Can Be Made

The issue of early clearance from the blood could be resolved through the coating of vector molecules with polymers, which could help avoid unnecessary interactions and block constitutive androstane receptor-mediated binding, facilitating evasion from unnecessary immune responses (Khare et al. 2011). Besides facilitation of evasion, the viral cells could also be modified to achieve targeting towards specific cells. For this purpose chimeric molecules have been developed, which allow both evasion from blood components and tissue sequestration, besides enhancing in vivo safety and retargeting of cells (Short et al. 2010).

Table 11.4 Advantages and disadvantages of Ad vectors (Appaiahgari and Vrati 2015; Gabitzsch et al. 2009; Shott et al. 2008)

Advantages	Disadvantages
Possess the ability to infect broad host range Induce high transgenic expressions without the integration of viral genes into host genome Stimulate toll-like receptor-dependent and receptor-independent pathways Present antigenic molecules to immune cells, with high efficacy by the virtue of increase chemokine and cytokine production, due to infection of dendritic cells Possess tropism for the epithelial cells, thus can be administered directly to mucosal and systemic immunity	HAd5 vector results in Ad-induced thrombocytopenia, and inflammatory responses, resulting in Ad-specific adaptive immune responses which could prove to be fatal Accumulate in liver and fail to transduce target cells, due to high affinity of hepatocytes to hypervariable regions of viral particles Tissue sequestration in other tissues of lungs, kidneys and spleen contribute to Ad-induced systemic toxicity Low oncolytic potential Reduced efficacy due to contamination with helper viruses

Adeno-Associated Vectors

Background

AAV is a non-enveloped virus, sized 20 to 25 nm, having single-stranded DNA as the genetic material. About 12 distinct serotypes are known for AAV named as 1–12, besides numerous recombinant species. AAV9 is known to have enhanced transduction capability for human cardiac, skeletal, liver, pancreas and eye tissue, with respect to other serotypes (DiMattia et al. 2012). AAV2 serotype possesses the unique property of site-specific integration in the mammalian DNA, thus assuming importance as the agent for gene therapy (Daya and Berns 2008). AAV's relationship with the human host is yet to be understood, as no correlation between viral infection and disease occurrence has been established yet. However, 80% of the human population exhibits antibodies against AAV serotypes 1–3 and 5 (Hüser et al. 2017). Several advantages and limitations of AAA in nucleic acid delivery have been summarized in Table 11.5.

Application for Nucleic Acid Delivery

AAV vectors find widespread application in gene therapy for diseases such as Parkinson's, Alzheimer's, cardiac disease, prostate cancer and other monogenic diseases, by the virtue of lack of pathogenicity, high persistence, and large number of serotypes (Ura et al. 2014). These vectors are also subjected to recombinant technologies such as trans-splicing, to increase their genome carrying capacity. AAV vector has also found application in gene delivery systems for treating cancer and exhibited strong safety profiles and high therapeutic efficacy. AAV vector also finds application in immunotherapy (Nieto and Salvetti 2014).

Table 11.5 Advantages and disadvantages of AAV vectors (Vannucci et al. 2013; Nieto and Salvetti 2014)

Advantages	Disadvantages
Derived from inherent replication-defective non-pathogenic virus	Possess limited capacity for transgene, can carry only up to 5 Kbp of heterologous DNA
Gutless vector does not code for any viral gene. High efficiency to perform in vivo transduction	Limited cloning capacity
Multiple capsids variants allow for avoidance of anti-capsids neutralizing humoral responses encountered after first injection	Widely persisting immunity in humans
	Require co-infection with a helper virus
	Difficulty in producing high titters

The AAV vaccines have been found to induce strong antibody responses even after single dosage, attributed to high and sustained transgenic expression. The sustained and continuous expression of antibodies introduced using AAV vector also makes them favourable agents of use for passive immunotherapy, wherein persistently high levels of antibodies are required to attain clinical efficiency (Nieto and Salvetti 2014).

Advantages and Disadvantages

Improvements that Can Be Made

The intrinsic immunogenic properties of the AAV vectors need to be enhanced in order to attain robust cytotoxic T-cell responses. The natural AAV serotypes are known to possess weak immunogenic profiles, due to which CD8+ T cell responses of low functionality are achieved. This could be overcome by either manipulating the vector genome to improve the kinetics and transgenic expression levels, or by changing the viral capsids which determine the tropism and transduction properties (Nieto and Salvetti 2014).

Retroviral Vectors

Background

Retrovirus is an enveloped virus, sized 80–100 nm, having single-stranded RNA as the genetic material, and contains reverse transcriptase enzyme. These are the replication defective vectors, requiring genome integration for gene expression, and are either avian or murine in origin. They also have low immunogenicity; however, those who contract diseases do not exhibit pre-existing immunity to this virus (Maurya et al. 2009). The retroviral transduction includes integration of viral genome into the hosts' genome resulting in stable genetic modification, which also forms the basis for

gene therapy (Ura et al. 2014). The retroviral vectors could either be replication defective or replication competent. These require a helper virus to support propagation (Maurya et al. 2009). However, the replication defective vectors are able to infect only a fraction of cells, whereas the replication competent vectors spread from cell to cell, and succeed in transducing the entire cell culture (Paar et al. 2007).

Advantages and Disadvantages

The major advantages and limitations of retroviral vectors in nucleic acid delivery listed in Table 11.6.

Application of Retroviral Vectors in Nucleic Acid Delivery

They act as agents of cell marking, for example in detection of malignant cell lines in human bone marrow (Vargas et al. 2016). These are used in gene therapy, for example in ADA treatment, wherein the blood cells transduced to express ADA and Npt genes are reinfused into the patient's body (Vargas et al. 2016). These viruses can also be used for gene delivery to target cells, for example CD4+ T cells in AIDS (Vargas et al. 2016).

Scope for Improvement

The replication-defective retrovirus vectors are able to infect only a fraction of cells; thus, attention is being given to replication-competent vectors. These vectors can spread from one cell to another, therefore delivering the gene of interest to large number of cells. However, the retroviruses are still associated with risks of oncogenicity and random integration, and low titers of self-inactivating derivatives. The vector particles could be modified to contain epigenetic regulators to facilitate the increased production, such as the matrix attachment region regulator (Buceta et al. 2011).

Table 11.6 Advantages and disadvantages of retrovirus vectors (Vannucci et al. 2013)

Advantages	Disadvantages
Vector genome integrates into host genome	Could bring about transduction of replicating cells only
Could carry up to 8 Kbp of heterologous DNA	Difficulty in achieving cellular targeting
Easy to engineer	Not suitable for quiescent cell populations
Wide range of tropism	Retroviral genome is randomly integrated into the host genome
Low immunogenic profile	High degree of risk for insertional mutagenesis
Absent or low pre-existing immunity	Low degree of stability
Easy production of high titters	

Lentiviral Vectors

Background

Lentivirus is closely related to retrovirus and constitutes a sub-class of the same. However, it is advance than retrovirus, as it possesses the ability to infect the quiescent cell populations as well and possesses even broader tropism. This allows the long-term availability of the therapeutic proteins and large construct libraries and facilitates ease in production (Sakuma et al. 2012). The safety concerns associated with the retrovirus are also existent with Lentiviruses, but the risks of tumorigenesis are much lower as the lentivirus integration sites are located away from the promoters (Ura et al. 2014). Different generations of lentiviral systems have been derived from HIV-1, out of which the third-generation system is widely used for clinical and research purposes (Merten et al. 2016). Lentiviral systems are used for gene therapy of rare genetic disorders and are also under development for treating the acquired diseases such as the haematological malignancies and serious infectious diseases (Kantor et al. 2014).

Advantages and Disadvantages of Lentiviral Vectors

The major advantages and disadvantages of lentiviral vectors are summarized in Table 11.7.

Application for Nucleic Acid Delivery (Hu et al. 2011; Singer and Verma 2008)

The major role played by lentiviral vectors is that they act as potent vehicles of gene delivery; thus they find different in vivo applications. Pertaining to their ability to infect the quiescent cells, the lentiviral vectors are used to infect particular sites in the brain to target the genes associated with neurological diseases. Lentiviral vectors are also used for performing in vitro cellular transduction, which makes them useful in generation of transgenic animals. Lentiviral vectors are used for immunization purposes for cancers and other infectious diseases as these are able to mediate efficient and long-lasting antigenic expressions. The antigens can be

Table 11.7 Advantages and disadvantages of lentiviral vectors (Vannucci et al. 2013)

Advantages	Disadvantages
Have the ability to transduce both quiescent and replicating cell populations	Possibility of insertional mutagenesis
Vector genome integrates into host genome	Packaging construct consists of regulatory proteins
Could carry up to 9 Kbp of heterologous DNA	Integration defective vectors results in transient transgenic expression
Facilitates prolonged gene expression	
Could be used as an integration defective vectors	

presented to dendritic cells both in vitro and in vivo resulting in activation of immune responses.

Scope for Improvement

Lentiviral vectors systems prove to be quite promising in devising new treatment methods and targeting new genes. It has been suggested that this vector system could be improved upon by increasing its versatility through expansion upon its genetic code. By making use of a variety of chemical moieties and identification of the potential modifiable sites, new rationales for designing of Lentiviral vectors could be devised, helping in advances tracking of virus and gene delivery mechanisms (Zheng et al. 2015). Also, better gene delivery systems, such as hydrogels, for the delivery of vector into the host could be adopted, to improve the stability of vector particles. The hydrogels could shield the vectors from responses of innate system, enhance transduction capabilities, and result in better retaining of the virus at the tissue site (Seidlits et al. 2013).

Herpes Simplex Virus Vectors

Background

Herpes simplex virus (HSV) exists as HSV-1(type 1) and HSV-2 (type 2), having large double-stranded DNA as genetic material. These viruses are characterized by their short reproductive cycles, establishment of latency in sensory ganglia, and immediate destruction of the host cell. The HSV vector is mainly derived from HSV-1, having the primary function of neuronal gene delivery, along the lines of parent virus' natural tropism. There are three types of HSV vector systems, the replication defective with deleted viral genes, attenuated replication incompetent, and the amplicon vectors, consisting of plasmids packaged into HSV particles (Lachmann 2004). The HSV vectors are able to infect both the quiescent and dividing cell populations, have high infectivity and transduction ability, hence pose as suitable candidates for gene transfer (Vannucci et al. 2013).

Advantages and Disadvantages

The major advantages and limitations of HSV vectors in nucleic acid delivery are listed in Table 11.8.

Table 11.8 Advantages and disadvantages of HSV vector source (Vannucci et al. 2013; Lachmann 2004)

Advantages	Disadvantages
Grows well in tissues cultures, thereby allowing retrieval of high titters Straightforward genetic manipulation Can accommodate large size foreign DNA, up to 50 Kbp Retrograde axonal transport allows targeting neurons located at distance from inoculation site Exists as episome in latent state therefore reducing the risks of integration in hosts chromatin	Production of safe vector genomes Tight transcriptional repression of viral genome in latent stage Transient transgenic expression Risk of recombination with latent HSV infected cells High pre-existing immunity in up to 70% of human populations

Application of Herpes Simplex Virus Vectors in Nucleic Acid Delivery

An important application of HSV vectors is for immunotherapy disabled infectious single cycle-herpes simplex virus (DISC-HSV) has been found to have specific application in vaccine against genital herpes. DISC viruses produce high level of transient gene expression before killing the transduced cells (Lachmann 2004). The HSV vector system is being utilized to protect the brain from ischemia, by identifying targets of the molecular pathways leading to neuronal death. The gene delivery systems are being designed to deliver therapeutic genes to such localized anatomical positions (Lachmann 2004). The neurons affected in Parkinson's relocalized to a single anatomical position. The HSV vector systems are being designed to deliver the therapeutic genes such as tyrosine hydroxylase, or expression of neurotrophic factors such as glial cell-line-derived neurotrophic factor (GDNF) to attain clinically efficient results (Lachmann 2004) However, the extension of such findings to human subjects is yet to be achieved.

Scope for Improvement

The HSV vectors are being tailored to increase their safety by removing immediate early genes, responsible for viral toxicity, from the genome. The amplicon-type HSV vector systems present immense potential as these are non-toxic and non-pathogenic and could carry a large amount of DNA. However, the replication-incompetent vector genome dilutes in the dividing cells resulting in failure of long-term gene expression. To attain long-term expression, the integration of transgenic cassette into chromosome of host cells has been suggested. The long-term expression could also be maintained by converting amplicon genome into replication competent extra chromosomal element (Esptein 2009).

Poxvirus Vectors

Background

Poxvirus is a large enveloped virus, having double-stranded DNA as the genetic material. These viruses have many genes (up to 250), and replication takes place in the entire cytoplasm of the host cell. The poxviruses are not the usual choice of preference due to a number of characteristics of their life cycle. However, these vectors find suitable application as expression vectors owing to high degree of stability of the freeze-dried vaccine accompanied with low costs and ease of manufacture. The vaccine can be administered through different routes, and a single inoculation possesses the ability to induce long-lasting antibody and T cell cytotoxic responses. Also, the poxvirus genome presents a high degree of flexibility, allowing removal of large amounts of genome and insertion of foreign DNA, allowing the creation of multivalent vaccines. Vaccinia virus can take up to 25 Kbp of DNA, making it useful for facilitating expression of large genes. The short lasting and intense expression of poxvirus vectors makes the poxvirus vectors desirable candidates for production of recombinant proteins, agents of cancer immunotherapy and vaccinations. The poxvirus immunizations have been designed against herpes virus, hepatitis B, HIV, influenza and others.

Advantages and Disadvantages

The major advantages and limitations of poxvirus vectors in nucleic acid delivery are listed in Table 11.9.

Scope for Improvement

The poxvirus vectors need to be reconsidered for use in gene therapy applications. The long-time usage of poxvirus vectors as highly immunogenic and effective vaccines has led to undermining its biotherapeutic potential. Vaccinia virus possesses the ability to maintain high transgenic expression and exhibits broad tropism. Also,

Table 11.9 Advantages and disadvantages of poxvirus (Vannucci et al. 2013)

Advantages	Disadvantages
Could carry up to 30 Kbp of heterologous DNA and offers multiple transgene insertion sites Apt as attenuated recombinant vaccine It faces low levels of pre-existing immunity	It is cytotoxic Generating recombinants is complicated Transient transgenic expression High degree of immunogenicity Difficulty in using heterologous promoters

the suggested use of poxviruses as non-replicating agents can affirm their utility in gene transfer studies as these can confer low risk of replication-associated toxicity.

11.6.2 Non-viral-Based Delivery Systems

Successful clinical trials of gene therapy over the past two decades have not achieved satisfactory expansion due to several limitations, mostly technical, one of the fundamentally important of which is the development of safe and effective delivery vehicles or 'vectors' (Kay 2011; Mingozzi and High 2011). Despite the substantial success, virus-based vectors have several drawbacks, including but not limited to carcinogenesis (Baum et al. 2006), immunogenicity (Bessis et al. 2004), difficulties in manufacturing (Thomas et al. 2003), DNA packaging (Bouard et al. 2009), and non-specific tropism (Waehler et al. 2007). Non-viral vectors have considerable potential to circumvent these limitations. Most commonly used non-viral vectors are relatively easy to synthesize and tend to possess other advantages including lower immunogenicity and higher genetic payloads than viral vectors (Pardi et al. 2017). Therefore, a range of synthetic delivery vehicles, including lipids and polymers, have received an impetus through the advancement of material science and deeper understanding of nucleic acid chemistry (Pack et al. 2005; Mintzer and Simanek 2009; Gonzalez et al. 1999; Love et al. 2010; Semple et al. 2010). with the simultaneous progress in nanotechnology, the scenario is changing rapidly as the biomedical community is ushered with a flood of newer delivery options, most of which are based on newer nanomaterials. In the following section, we have summarized different kinds of nanomaterial-based non-viral vectors, challenges and potential uses of them for in vivo delivery of therapeutic nucleic acids.

Advantages of Using Nanoparticles for Nucleic Acid Delivery

Nucleic acids can be delivered by coating over or loading inside the nanoparticles which offer a wide range of benefits as: (i) they can be in the size range suitable for uptaking the cells by its routine uptake mechanisms such as endocytosis; (ii) they can be synthesized using various methods and materials which can be tailored to individual vaccine types and (iii) they have ability to form stable conjugates and slow controlled release of vaccines.

Types of Nanoparticle-Based Systems

Despite remarkable success, gene-based therapeutic modules still need serious attention in order to curb down the potential toxicities, mainly associated with their delivery strategies. The application of nanoparticles for successful gene therapy has emerged as efficient and safe delivery of genes to the target cells. Besides safe

delivery, nanoparticles also contribute to theranostics, an integrated platform of therapeutics and diagnosis, to simultaneous monitor the gene delivery and response of the modified cells to the undergoing treatment (Jeelani et al. 2014; Xie et al. 2010). Here we will briefly describe some nanoparticles, having significances in gene therapy.

Magnetic Nanoparticles

Among the wide spectrum of inorganic nanomaterials, magnetic nanoparticles (MNPs) have received special attention because of their intrinsic physical properties which serve both the diagnostic as well as therapeutic purposes. MNP-based therapeutics not only mediate efficient target-specific drug/gene delivery but also confer an additional advantage of monitoring real-time systemic response to the therapy (Koenig and Kellar 1995). Majority of MNPs used in biomedical applications are iron oxide based. Often, doping these iron oxide nanoparticles with high magnetic moment elements, for example Mn, Co, Ni though can give superior MRI contrast agents but they are highly toxic, limiting their use in clinical applications (Kami et al. 2011). However, iron oxides such as magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3), in particular are relatively non-toxic, and thus, widely used in the form of superparamagnetic nanoparticle core in several biomedical applications (Weissleder et al. 1989). Iron oxide nanoparticles (IONPs) are prepared by one of the following methods: flame-spray pyrolysis, co-precipitation, solvothermal/hydrothermal synthesis, micro-emulsion and high-thermal decomposition (Gupta and Gupta 2005; Schladt et al. 2011; Akbarzadeh et al. 2012). Like other nanoparticles, the design of MNPs also needs to observe certain parameters in order to be successful therapeutic delivery vehicle. These parameters are as follows:

1. *Morphology*: It has been studied that anisotropically shaped MNPs have several advantages than spherical MNPs, such as prolonged circulation in blood, longer period of retention in tumor, large surface area, multisegmented attributes etc (Liu et al. 2007a; Fratila et al. 2015). J.H. Park et al. evaluated that magnetic iron oxide nanoworms with higher aspect ratio (more elongated shape) are able to spend more time in blood circulation than their spherical counterpart (Park et al. 2009). Recently, C. E. Smith et al. developed worm-like elongated superparamagnetic iron oxide nanoparticles' (SPIONs) clusters which have tenfold higher binding affinity to the target substrate than spherical ones (Smith et al. 2017). Moreover, iron oxide-based MNPs with higher aspect ratio contribute to generating enhanced hyperthermia and better contrast in magnetic resonance imaging (MRI) applications in comparison to spherical analogs (Das et al. 2016).
2. *Physicochemical properties and superparamagnetism*: Navigating through the systemic circulation to the target site and efficient release of drugs requires the nanoparticles (NPs) to trespass all the biological barriers which include reticuloendothelial system (RES), non-specific interaction with undesired proteins in plasma, lysosomal trafficking inside the target cells etc. Moreover, for effective

gene therapy, an additional barrier NPs have to face is the nuclear membrane (Schladt et al. 2011). If these will remain unaddressed, then they will reduce the biodistribution, duration of systemic circulation time of NPs, and inadequate release of therapeutic payloads from them. Therefore, efforts have been put to understand the physiochemical properties of MNPs, including morphology, hydrodynamic size, surface properties and how different surface modifications can augment their therapeutic standard. All kinds of surface modification of MNPs usually result in a core-shell-like structure, with IONPs being the core and a biologically inert material being the protecting/stabilizing shell. In order to be used for biomedical purposes, magnetic materials should be super-paramagnetic as they show zero residual magnetization and coercivity in the absence of external magnetic field that in turn helps in avoiding coagulation under in vivo condition (Belting et al. 2005; Chouly et al. 1996).

3. *Hydrodynamic size*: MNPs of >200 nm are filtered mechanically or by phagocytosis in the spleen (Moghimi 1995) and NPs of <10 nm are seen to be subjected to rapid renal clearance (Liu et al. 2013; Choi et al. 2007). The ideal size of MNPs is preferred to be within 10–50 nm. Smaller sizes offer several advantages, for example, higher diffusion rates, prolonged circulation, larger effective surface areas, more colloidal stability and more resistance to agglomeration, etc. With reduction of size, coercivity initially increases to maximum, but then decreases to zero as in case of superparamagnetic NPs (Guo et al. 2013). SPIONs, due to their very small core size, can exist in multiple discrete domains which increase their magnetic saturation and susceptibility to external magnetic field. Size also determines the biodistribution of MNPs. Lot more research works are still required in order to gain a substantial knowledge about the role of hydrodynamic sizes on MNPs' activity.
4. *Surface properties*: In addition to morphology and sizes, surface properties are also crucial for determining the pharmacokinetics of MNPs, including biodistribution, absorption, metabolism, toxicity, excretion, etc., and over the last two decades, different modifications of surface chemistry have been studied with the aim of designing MNPs possessing lesser toxicity, enhanced biocompatibility, higher therapeutic or genetic payloads, etc.

These surface modifications usually include small organic molecules or biocompatible polymers which function to (1) prevent MNP aggregation; (2) restrict drug tropism to the target sites; (3) prevent premature release of drugs and its degradation; (4) create docking sites for conjugating drug molecules, targeting ligands; and (5) increase overall therapeutic efficacy. A wide variety of polymer coating, such as dextran, chitosan, polyethylene glycol (PEG), poly (D, L-lactic-co-glycolic acid) (PLGA), polyethylenimine (PEI), polyvinylpyrrolidone (PVP), polyaniline, and organic surfactants, such as sodium oleate and dodecylamine have been investigated for surface coating purpose (Guo et al. 2013; Gamucci et al. 2014; Singh and Sahoo 2014). Besides these, inorganic molecules such as silica (Agotegaray and Lassalle 2017), gold (Arsianti et al. 2011; Moraes Silva et al. 2016) and gadolinium (Santra et al. 2012) are often used to MNP surface modification. Figure 11.5 shows a gen-

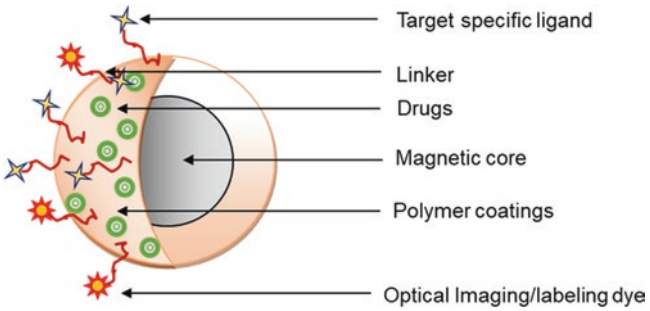


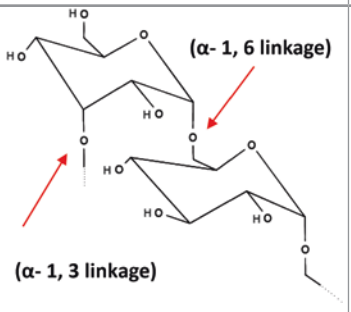
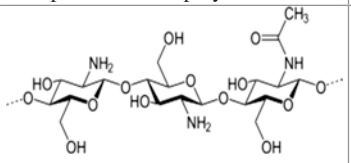
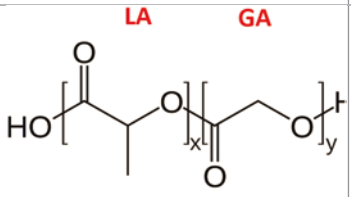
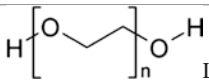
Fig 11.5 Schematic representation of surface functionalized magnetic nanoparticle where magnetic core is coated with a biocompatible polymer containing site-specific targeting ligands or optical imaging/labelling dyes and therapeutic drugs

eralized scheme for different types of nanoparticle surface functionalizations, and Table 11.10 discusses different surface coating polymers.

Surface coating may also comprise more than one polymer in order to take the benefits of distinct functionalities of individual polymers. For instance, Kievit et al. developed a SPION-based DNA carrying nanovector coated with a copolymer of PEG-g-chitosan-g-PEI where the superior DNA transfection efficiency of PEI is combined with efficient stabilizing and immunoevasive property of PEG and chitosan helps in lowering the cytotoxicity (Kievit et al. 2009). Elham et al. developed a copolymer of poly(ethylene glycol)-poly(lactic-co-glycolic acid) (PEG-PLGA) as a nanocarrier platform for delivery of an anticancer flavonoid drug, 'chrysin' in breast cancer cell line T47D. PEG-PLGA copolymer provides the necessary stabilization, enhances circulation time and was shown to increase cytotoxicity in cancer cells particularly (Anari et al. 2016). Lo et al. developed chondroitin sulphate-PEI copolymer-coated SPIONs (CPIOs) as a highly efficient 'transgene' nanocarrier for delivery of miR-128 encoding plasmid into mammalian cells (Lo et al. 2015).

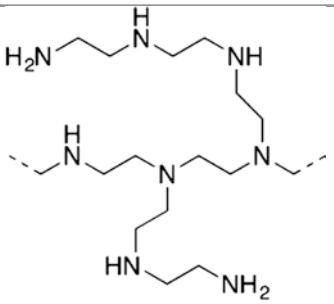
5. *Targeted delivery*: Conventional chemotherapy has several inherent shortcomings including non-specific action, rapid clearance, immune rejection etc. To limit off-target effects, nanoparticle surfaces have been engineered to provide them specific affinity to target tissues. 'Passive targeting' is one such strategy which was studied mostly for cancer therapy (Singh and Sahoo 2014; Bazak et al. 2014). Here, NPs are formulated by taking the advantage of leaky neovasculature and their inefficient drainage system of solid tumor tissues which results in enhanced permeation and retention (EPR) effect of NPs (Maeda 2010). Owing to inefficient internalization of NPs, passive targeting is limited for only certain in vivo applications, and therefore, significant amount of efforts have been put also on decorating the MNP surface by different target-specific ligands which are tethered to MNP surface or on the polymer coating by several functional groups or chemical linkages such as amine ($-NH_2$), sulfhydryl (-SH), carboxyl (-COOH), pyridyl disulphide linker, carbodiimide/hydroxysuccinimide (EDC/NHS) linker

Table 11.10 Different types of coating polymers used for MNP core surface fabrication

Polymer	Structure	Properties	Advantages
Dextran	 Complex branched polysaccharide	1. Biocompatible 2. Hydrophilic	1. Non-immunogenic 2. Enhancement of blood circulation time 3. Stabilization of NPs in acidic and basic environment 4. Protection from nuclease (Unterweger et al. 2018)
Chitosan	 Linear polysaccharide composed of β-(1 → 4)-linked D-glucosamine and *N*-acetyl-D-glucosamine	1. Biocompatible, biodegradable 2. Cationic and hydrophilic 3. High charge density 4. Mucoadhesive	1. Non-immunogenic 2. Lower cytotoxicity (Puri et al. 2009) 3. Controlled release of encapsulated drug, prolonged accumulation of therapy (Kim et al. 2008) 4. Improved absorption at cell surface (Kim et al. 2008; Choi et al. 2004a)
Poly (D, L-lactic-co-glycolic acid) (PLGA)	 Linear polymer with a polyester backbone of lactic acid (LA) and glycolic acid (GA)	1. Biodegradable (hydrolysed into monomer easily) 2. Hydrophobicity and crystallinity can be manipulated by varying ratio of LA and GA.	1. Non-immunogenic 2. Non-cytotoxic 3. Controlled release of encapsulated drug, prolonged accumulation of therapy (Gamucci et al. 2014) 4. Stabilize encapsulated drugs or nanoparticles
Polyethylene glycol (PEG)	 Linear polyether	1. Biocompatible 2. Mostly hydrophilic 3. Impart water dispersity	1. Non-immunogenic 2. Functionally suitable to avert capturing of coated MNPs by RES ('STEALTH' MNP) (Li and Huang 2010) 3. Prevention of ROS induction by iron oxide nanoparticle 4. Non-cytotoxic 5. Reduce non-specific protein binding to NPs (He et al. 2010) 6. Protection from nuclease.

(continued)

Table 11.10 (continued)

Polymer	Structure	Properties	Advantages
Polyethylene-imine (PEI)	 Linear or branched polymer composed of repeating units of amine group and $–CH_2CH_2$ spacer	1. Non-biodegradable 2. Hydrophilic 3. High charge density	1. Non-immunogenic 2. Strong electrostatic conjugation with siRNA or DNA facilitates their efficient delivery; High transfection efficiency (Jin et al. 2014) 3. Proton sponge effect: Proton flux-based osmotic swelling of endosome and subsequent rupture leads to drug release to target cell (Creusat et al. 2010; Guo and Huang 2011; Hwang et al. 2001). 4. Shows variable cytotoxicity depending upon molecular weight, size and degree of branching (Wightman et al. 2001; Godbey et al. 1999).

and physical interactions such as electrostatic binding, hydrophobic interaction, etc. (Agotegaray and Lassalle 2017) A varying number of ligand guided MNPs can deliver drugs site specifically leading to enhanced therapeutic efficacy. Table 11.11 describes several examples for each type of targeting ligand. It is worth mentioning that some of these agents contribute the dual purposes, such as chlorotoxin which serves both as a therapeutic drug and a targeting ligand in brain tumor (Sun et al. 2008a; Sun et al. 2008b; Veiseh et al. 2009). In addition, optical imaging dyes often are attached to the surface of the nanoparticles (Chekina et al. 2011; Nickels et al. 2010). Thus, surface functionalization of MNPs plays a crucial role in determining the therapeutic fate of encapsulated drugs and their efficacy.

Recent Advances in Magnetic Nanoparticle-Mediated Gene Delivery: Magnetofection

Magnetofection is a method of intracellular delivery of nucleic acids under the influence of external magnetic field acting on nucleic acid vectors associated with magnetic nanoparticles. A PEI-coated magnetic nanoparticle system developed by Scherer et al. is the first example of in vitro non-viral MNP-mediated gene delivery (Scherer et al. 2002). Since then a number cell lines including primary lung epithelial cells (Gersting et al. 2004) and blood vessel endothelial cells (McBain et al. 2007) has been transfected by magnetofection epithelial cells and blood vessel endothelial cells. McBain et al. developed PEI-coated magnetic particles by covalently coupling PEI to the surface of composite iron oxide-dextran silica particles

Table 11.11 Molecular targeting strategies using magnetic nanoparticles: Different targeting ligands, their cellular targets, application and references

Type of molecules	Example	Target	Application/functional activity	References
Small molecules	Folic acid	Folate receptor	Breast cancer, prostate cancer, facilitate cellular uptake of nanoparticles	(Sun et al. 2006; Luo et al. 2017)
Peptides	RGD	$A_v\beta_3$ integrin	Breast cancer	(Montet et al. 2006; Lee et al. 2009)
	Chlorotoxin	MMP2	Brain tumor imaging, glioblastoma therapy	(Li and Huang 2010; Sun et al. 2008a)
Proteins	Transferrin	Transferrin receptor	Breast cancer imaging	(Kresse et al. 1998)
Aptamers	A10 aptamer	Prostate-specific membrane antigen (PSMA)	Prostate cancer	(Wang et al. 2008)
	MUC1	MUC1 receptor	Ovarian cancer	(Shahbazi-Gahrouei and Abdolahi 2013; Azhdarzadeh et al. 2016)
Antibodies	Trastuzumab (HER2 antibody)	HER2 receptor	Breast cancer, prostate cancer	(Cirstoiu-Hapca et al. 2007)
	Rituxan (rituximab)	CD20 antigen (B-cell non-Hodgkin lymphoma)	Lymphoma imaging	(Funovics et al. 2004)

using glutaraldehyde linkers (McBain et al. 2007). Recently, Pickard MR et al. developed magnetic nanoparticle-mediated gene delivery to two- and three-dimensional neural stem cell cultures (Pickard et al. 2017). Magnetic nanoparticles are also used for anticancer combinatorial therapy where siRNA molecules are co-delivered with standard conventional chemotherapeutic agents. RNAi alone results in a partial or ephemeral anticancer gene silencing whereas when combined with a chemotherapeutic drug their anticancer effects become much more dominant. Both the siRNA and the chemotherapeutic drug can act exclusively or synergistically. Guruprasath P et al. designed an IL4RPep-1 (IL-4R-targeting peptide)-conjugated bPEI-SPION carrying Bcl-xL siRNA to MDA-MB231 breast tumor cells which makes them more responsive to doxorubicin, a potential anticancer drug, compared to untargeted BPEI-SPION/Bcl-xL siRNA (Guruprasath et al. 2017). Along with this, a potentially emerging area of interest associated with SPIONs is their optical property due to which they are exploited as high contrast agent for magnetic resonance imaging, helping in evaluating the real-time response of drug or gene treatment via them. Li D et al. developed a bioreducible polyethylenimine-coated iron oxide nanoparticle (SSPEI-SPIO) capable of reduction-induced gene delivery at target sites and magnetic resonance imaging (Li et al. 2014).

Lipid-Based Nanoparticles

Since its inception, studies on non-viral vectors mediated delivery of drugs are mostly aimed at increasing their therapeutic quotient while reducing their side-effects. Recent development and understanding of nanoparticles' chemistry and interaction with biomolecules have triggered an exponential growth of their versatile application as pharmaceutical drug carriers. Among other non-viral vectors, cationic lipid nanoparticles gained substantial attention for mostly their ability of packaging different therapeutic drugs into a biologically inert and non-toxic environment and thus can transport them safely as a single delivery system (Yin et al. 2014; Love et al. 2010; Semple et al. 2010; Shim et al. 2011, 2013; Xiong et al. 2011). Therefore, though they have been introduced almost two decades ago, cationic lipids remain one of the major choices of lipid-based carriers for gene delivery. In recent years, cationic lipid-based nanoformulations have overcome several technical challenges and have been shown to successfully deliver therapeutic drugs, including bioactive molecules, nucleic acids (siRNA, miRNA), etc. (Love et al. 2010; Semple et al. 2010; Shim et al. 2011, 2013; Xiong et al 2011; Sarker et al. 2013, 2012; Aoshima et al. 2013). A versatile group of cationic lipid-based nanocomplexes, for example, liposomes, ionizable lipid lipids, lipid nano-emulsions and solid lipid nanoparticles, have been studied for nucleic acid and protein delivery. The chemical structures of few these groups were shown in Fig. 11.6. Here, we will discuss about them and their various applications briefly.

Liposomes

Among various lipid-based nanoparticles, 'liposomes' are classical examples. Bangham AD et al. first developed the liposomes with encapsulated solutes (Bangham and Horne 1964; Bangham et al. 1974). In 1987, Felgner et al. first studied a highly efficient method of lipid-mediated DNA transfection (lipofection) where small unilamellar liposomes were prepared from *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) containing a monovalent cationic head and two hydrocarbon tails and they were used to deliver DNA in mouse L cells (Felgner et al. 1987). Since then, various cationic lipids and their efficiency in the form of lipid-based nanoparticles have been evaluated, for example, (±)-N,N-dimethyl-N-[2-(sperminecarboxamido) ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA), N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulphate (DOTAP), N′,N′-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide (DODAG) and 3β [*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Cholesterol) (Yin et al. 2014; Tam et al. 2013; Kanasty et al. 2013; Sato et al. 2012; Hafez et al. 2001). Most of these newly synthesized lipid molecules showed excellent DNA complexation capability. Mevel et al. showed DODAG transferred plasmid DNA efficiently and the transfection efficiency in OVCAR-3 and HeLa cell lines is more than lipofectamine2000 (Mevel et al. 2010). Cationic liposomes often also incorporate neutral lipids as 'helper' molecules in order to increase liposomal stability and facilitate transfection (Yin et al. 2014). For example, the neutral lipid, 1,

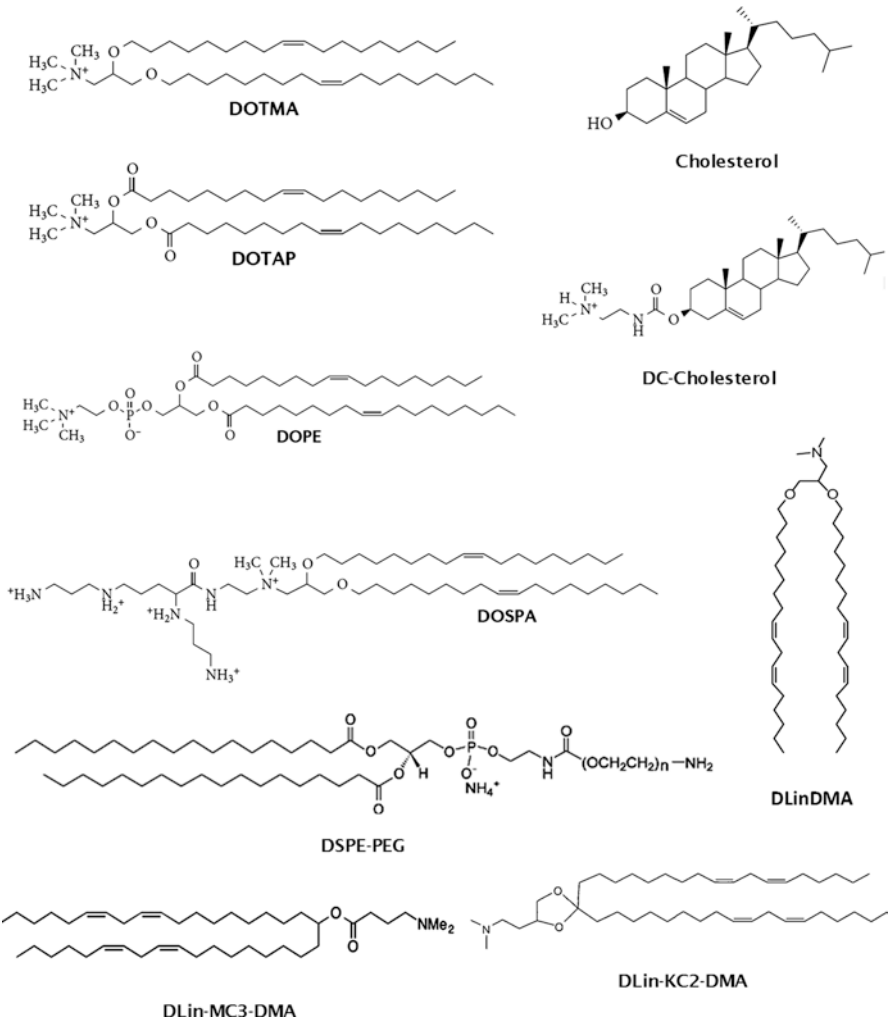


Fig. 11.6 Chemical structures of several cationic, ionizable and neutral lipids. Cationic lipids (such as DOTMA, DOTAP, DC-Cholesterol) are able to bind strongly DNA molecules and thus have an active role in transfection. DLin-MC3-DMA and DLin-KC2-DMA are two famous examples of ionizable lipid. Neutral lipids (such as DOPE, cholesterol) function mainly by stabilizing cationic lipid nanoparticles and enhancing overall transfection efficiency

2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) is known to facilitate endosomal escape of lipoplexes (Chen et al. 2011). Mochizuki et al. reported that cationic lipid bearing ethylene diamine shows better transfection efficiency and reduced cytotoxicity when combined with DOPE (Mochizuki et al. 2013). The advantages of using DOPE are believed to be associated with its structural changes, from micelle to an inverted hexagonal (H_{II}) state at acidic pH which facilitates the particle in endosomal escape. Similarly, often cholesterol, another neutral lipid also

is inserted into liposome as 'helper' lipid to enhance the rigidity of the system (Mosca et al. 2011). Cholesterol also provides protection to oligonucleotides from degradation and helps lipid particles to interact with cell membranes (Tam et al. 2013; Kanasty et al. 2013; Sato et al. 2012; Hafez et al. 2001; Chen et al. 2010a).

Cationic liposomes have been extensively studied for different gene delivery purposes. Y Chen et al. developed a systemic and targeted delivery strategy of siRNA and miRNA by a ligand guided PEGylated cationic liposome into an experimental lung metastasis model of murine B16F10 melanoma. They encapsulated a therapeutic hyaluronic acid modified and protamine conjugated siRNA or miRNA directly into a PEGylated and tumor-specific ligand (GC4 ScF) modified cationic liposome prepared from DOTAP/cholesterol (1:1 mol/mol) (Chen et al. 2010a). This nanoencapsulated formulation showed excellent systemic delivery and tumor suppression. R Sheng et al. developed a co-assembly of diosgenin-based cationic lipid (Dioasrg) and DOPE which showed improved intracellular delivery of both siRNA and plasmid DNA (Sheng et al. 2016). Cationic liposomes have delivery of antisense oligonucleotides (AONs). Cationic 'elastic' liposomes, an improved liposome structure, made from DOTAP and sodium cholate was studied by S T Kim et al. for the delivery of AONs binding interleukin 13 (IL-13) as a therapeutic strategy for the treatment of atopic dermatitis (Kim et al. 2009). Along with these, therapeutic delivery of miRNA via cationic lipid-based lipoplexes has also been studied. For example, Y Wu et al. have shown successful delivery of miR29-b in A549 cells (non-small lung cancer cells), by a cationic liposome comprising of DOTMA as the building block, cholesterol and D-α-tocopheryl polyethyleneglycol 1000 succinate (vitamin E TPGS), a short poly(ethylene glycol) (PEG) molecule linked to vitamin E. This nanoencapsulated miRNA displayed effective reduction of the expression of its cellular target gene CDK6 (Wu et al. 2013a).

Ionizable Lipids

Ionizable lipids are an advanced tool of lipid-based non-viral gene delivery which has a unique capability to modulate its surface charge depending on the pH of the surrounding environment. The behaviour made them more suitable nucleic acid career as they are less toxic and can swiftly perform endosomal escape. In order to minimize toxicity without reducing efficacy, ionizable lipids should have a pK_a value lower than the pH of the physiological environment but higher than the pH of endosomal environment because only then they will be able to remain unprotonated in circulation and upon internalization their amine groups will become protonated, thus facilitating their association with anionic endosomal lipids which in turn will help further for endosomal escape. An additional advantage of their physicochemical property is, when in circulation, due to the neutral state, they can easily dupe RES mediated clearance and thus improve circulation time (Semple et al. 2001). Mainly the structure of the hydrophobic tails of the lipid molecules determines both the pK_a and the efficacy. 1,2-Dioleoyl-3-dimethyaminopropane (DODAP) is the first ionizable amino lipid with pK_a of 6.6–7 and one double bond in each of its acyl tail that was used to be loaded with nucleic acids (Tam et al. 2013; Kanasty et al. 2013; Sato et al. 2012; Hafez et al. 2001; Semple et al. 2001). Two most efficacious

ionizable lipids, DLin-KC2-DMA (2, 2-dilinoleyl-4-(2-dimethylaminoethyl)-[1, 3]-dioxolane) (Semple et al. 2001) with a pK_a of 6.7, and DLin-MC3-DMA (1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane) (Jayaraman et al. 2012) with a pK_a of 6.4, have been successfully used to develop stable nucleic acid lipid particles (SNALPs) which are 100-fold and 1000-fold more efficient in inhibiting hepatic genes than the previous generation lipid DLin-DMA (1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane). ALN-TTR02, a lipid nanoparticle encapsulated transthyretin (TTR) siRNA studied for the treatment of transthyretin-mediated amyloidosis (ATTR), currently in phase II clinical trial (Alnylam Pharmaceuticals; ClinicalTrials. gov ID: NCT01617967) consists of DLin-MC3-DMA as core lipid shell (Schladt et al. 2011).

Lipid Nanoemulsions

Emulsions are fine dispersions of an immiscible liquid onto another, stabilized by a third compound, the emulsifying agent. One class of emulsifying agent is known as 'surface active agent' or 'surfactant'. When cationic lipids are used as surfactant molecules, they get distributed at the interface between the inner oil and the outer aqueous phases, and these dispersed systems can work as a suitable gene delivery vehicle. Cationic emulsions consisting of cationic lipids and core oil have several advantages which make them superior gene delivery candidate. While cationic liposomes form larger aggregates at higher concentration and become unstable in serum due to their surface charge which leads to their rapid clearance by RES, cationic lipid nanoemulsions (cLNE) show long-term stability in bloodstream owing to smaller sizes and their surfaces are barely recognizable by RES. Other advantages of cLNE include toxicological safety, higher content of lipid phase, increased drug loading, controlled drug release and cost-effective method of large scale preparation (Nam et al. 2009). cLNE also incorporates 'helper' lipid DOPE, which, due to their fusogenic property, provide extra stability in the carrier system and improve overall transfection efficiency. For example, Choi et al. prepared cLNE of varying composition of DC-Cholesterol, DOPE, castor oil and Tween 80. They showed one cationic emulsion (E2; DC-Cholesterol/DOPE/Castor Oil/Tween 80 = 0.3:0.3:0.3:0.15) has better stability, stronger serum resistance, prolonged circulation and better transfection efficiency than a cationic liposome (L3; DC-Chol/DOPE = 0.6:0.3) (Choi et al. 2004a). In addition, to avoid detection by immune cells and serum stabilization, like other nanoparticles emulsions are also modified with PEG which makes them more suitable for systemic delivery of therapeutic nucleic acids. Fraga M et al. synthesized a PEGYlated cLNE from medium chain triglycerides (MCT)/DOPE/DOTAP/DSPE-PEG (1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000]) by high pressure homogenization which can efficiently encapsulate preformed plasmid DNA (encoding α-L-iduronidase)-DOTAP complexes into its oil core. This PEGylated nanoemulsion also displayed efficient transfection of the plasmid DNA when systemically administered (Fraga et al. 2015). Nonetheless, some research groups showed a concern for lowering cytotoxicity of this type of assembly. Therefore, newer cationic lipids with two alkyl tails, aspartate or gluta-

mate backbone or a lysine head-group have been suggested which exhibited lesser toxicity without compromising the ability of gene delivery (Obata et al. 2008). In addition to these, amino acid modified fatty acids such as lauroyl-arginine methyl ester have been developed which were shown to have great self-assembly property, biocompatibility and low toxicity (Pinazo et al. 2000). Despite several advantages, only few research examples exist where lipid nanoemulsions are used as a delivery system for RNAi. For example, Kaneda et al. used cLNE containing DOTAP, DOPE and cholesterol for siRNA delivery. In this report, transfection complex of ~300-nm size showed significant suppression of upregulated vascular adhesion molecules by endothelial cells (Kaneda et al. 2010). Delivery of AONs by cLNE has also been studied. For example, Bruxel et al. prepared a DOTAP-based nanoemulsion system for the delivery of AONs targeting malarial topoisomerase II (Bruxel et al. 2011). Recently, Brito LA et al. reported the delivery of self-amplifying mRNA vaccine (an mRNA sequence encoding not only the antigene of interest but also a viral RNA-dependent RNA polymerase which helps to amplify the mRNA in the cytoplasm of the transfected cells) by a cLNE composed of cationic lipid DOTAP emulsified with the constituents of the emulsion adjuvant MF59. The article showed that the self-amplifying mRNA delivered via CLNE is well tolerated and capable of stimulating strong immune response in the chosen animal models (Brito et al. 2014).

In recent years, use of solid lipid nanoparticles as nucleic acid delivery system has become a popular alternative of liposome mediated transport. Solid lipid nanoparticles are typically spherical nano carrier system with a solid lipid core matrix (Wissing et al. 2004). Solid lipid nanoparticles, prepared from cationic lipids, have been actively preferred to be used for successful gene delivery because of their possible electrostatic interaction with the negatively charged nucleic acid. Carrillo et al. showed how cationic solid lipid nanoparticles (cSLNs) form complexes with plasmid DNA and deliver them (Carrillo et al. 2013). The other unique properties which give this nanoparticle system distinct recognition include: (1) high drug loading, (2) larger surface area, (3) ability to protect the drug from the environment, (4) no organic solvents for their assembly and (5) their low-cost production process.

Solid Lipid Nanoparticles

Moreover, drug mobility decreases in the solid lipid phase which leads to the better-controlled release of encapsulated drugs (Puri et al. 2009; Basaran et al. 2010). cSLNs are usually prepared by various methods, including high-pressure homogenization, microencapsulation, phase inversion and the solvent injection technique (Puri et al. 2009). Use of cSLNs in siRNA delivery has also shown promising results. For example, Kim et al. developed a cSLN-based nanocarrier from cholesteryl ester, triglyceride, cholesterol, DOPE and DC-chol which were electrostatically bound to PEGylated DNA. This siRNA nanocomplex showed comparable gene silencing efficiency in PC3 and MDAMB435 cell lines (Kim et al. 2008).

Polymer-Based Nanoparticles

We already have discussed different polymers as a surface coating material for different nanoparticles. Apart from being a material for surface coating of different nanosized gene delivery vehicles, polymers themselves have been studied as a carrier of genetic material. Cationic polymers have gained popularity as a promising nucleic acid carrier because of their capability of forming association with DNA/RNA via electrostatic bonding and high transfection efficiency (Jin et al. 2014). Currently, many natural and synthetically prepared cationic polymers are being investigated for gene delivery purpose. Due to their biocompatibility and biodegradability, cationic polymers, such as chitosan, derivatives of dextran (e.g. DEAE-dextran), collagen (e.g. atelocollagen), gelatin and cyclodextrin, have been favoured as nucleic acids delivery agents (Nitta and Numata 2013; Draz et al. 2014). Among synthetic polymers, Poly (L-lysine) (PLL) and polyethylenimine (PEI) are the two early members who stirred substantial interest as suitable gene carrier (Yin et al. 2014). Recently, a biocompatible tri-block copolymer PEO20-PPO69-PEO20 as a gene delivery vector [PEO, poly(ethylene oxide); PPO, poly(propylene oxide)] has shown promise (Daima et al. 2018). The study of polymers for gene transfer gained a splendid attention and remained globally popular for a long span of time around the 1960s and 1970s. For example, the ability of PLL to condense DNA was known since the 1960s (Olins et al. 1967; Laemmli 1975). Wu et al. constructed an asialoorosomucoid glycoprotein (specific ligands for hepatocytes' receptors) coupled PLL-based soluble DNA carrier which showed strong binding to negatively charged plasmid DNA, pSV2 CAT (encoding chloramphenicol acetyltransferase) and their efficient transformation into the target sites (Wu and Wu 1987; Wu and Wu 1988). Polymer-based gene delivery though has a long history, but the active role of PEI in gene transfer got revealed at the end of the previous century when Boussif O et al. first established the role of PEI as a promising vector for gene therapy (Boussif et al. 1995). Soon after that, transfection efficiency and cytotoxicity of PEI and several of its variants have been evaluated (Wightman et al. 2001; Godbey et al. 1999). A great length of efforts have been put to develop newer variety of synthetic polymers with higher transfection efficiency and lesser toxicity such as polycationic dendrimers such as poly(amidoamine) (PAMAM) (Choi et al. 2004b; Kesharwani et al. 2014), poly [(2-dimethylamino) ethyl methacrylate] (pDMAEMA) (Li et al. 2013) and poly (β-amino ester)s (Yin et al. 2014; Zhou et al. 2011). Few of the chemical structures of cationic polymers were shown in Fig. 11.7.

With the progress of nanotechnology and improved understanding of nucleic acid chemistry, although a versatile range of sophisticated nanocarrier systems for gene delivery have been developed and studied, the cationic polymers still offer a wide variety of advantages like wide chemical diversities, greater stability, strong electrostatic binding with encapsulated nucleic acid and relatively easier method of functionalization etc., due to which they still are at the centre of attention (Jin et al. 2014; Kesharwani et al. 2014; Li et al. 2013; Zhou et al. 2011; Wang et al. 2012). In addition to these, cationic polymers generally do not contain hydrophobic core and thus are more soluble in water than lipid-based nanocarriers. Moreover, they can

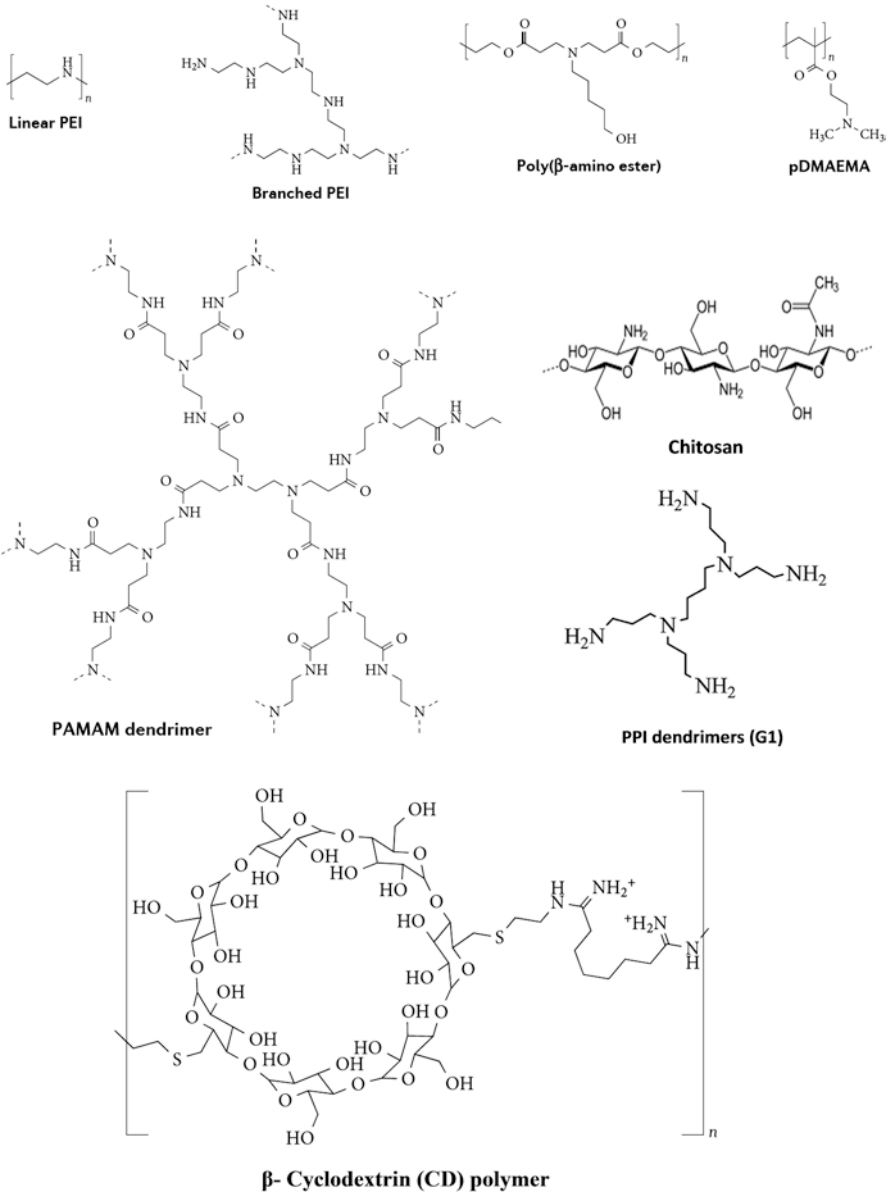


Fig. 11.7 Chemical structures of different cationic polymers. Among them chitosan is the natural biopolymer, and others are synthetically prepared. PEI is the most extensively studied polymer showing high transfection efficiency. Other polymers also have various advantages

confine nucleic acids into a very small area than cationic liposome. Here, we briefly review some of these essential polymers and their use for gene delivery.

Chitosan

Chitosan is one of the natural cationic polymers, widely used for gene delivery. Chitosan is a linear cationic polymer of N-acetyl-d-glucosamine and D-glucosamine linked by β-(1–4) glycosidic bonds and is prepared by partial deacetylation of chitin (a fibrous, polysaccharide found in the shells of crustaceans) in presence of sodium hydroxide (Yen et al. 2009). This polymer ($pK_a = 6.2–7$) though is insoluble in alkaline and basic medium, but their amine groups become positively charged in acidic pH values which impart them an overall highly dense cationic character. This property helps in efficient encapsulation of nucleic acids and the successful endosomal escape (Liu et al. 2005). The versatile application of chitosan and its functionalized derivatives stem from its natural abundance, low toxicity, biocompatibility and biodegradability and higher degree of surface absorption (mucoadhesiveness) which further facilitates proper cell internalization of nanoparticle encapsulated nucleic acid (Rao and Sharma 1997; Aspden et al. 1997; Xu et al. 2010; Sarmento et al. 2007; Roldo et al. 2004).

Thorough investigations revealed that apart from surface charge molecular weight (Sato et al. 2001; MacLaughlin et al. 1998; Ishii et al. 2001), nitrogen to phosphate charge ratio (N/P) (Sarmento et al. 2007; Roldo et al. 2004; Sato et al. 2001) and degree of deacetylation (Huang et al. 2005; Lavertu et al. 2006) of chitosan polymer play crucial role in determining both the drug/gene encapsulation efficiency and their successful delivery. Studies revealed that the encapsulation efficiency increases with the degree of deacetylation (Huang et al. 2005; Lavertu et al. 2006). Numerous studies have been conducted where chitosan has been used to complex with nucleic acids and successfully transfer them inside the cell. Chen J et al. complexed chitosan with plasmid DNA containing mEpo gene forming chitosan-DNA nanoparticles which were stable when orally administered and efficiently transfected into in intestinal epithelia (Chen et al. 2004). Jean M et al. developed a chitosan-plasmid DNA nanocomplex encoding glucagon-like peptide 1 (GLP-1; used for treatment of type 2 diabetes) and on subsequent successful delivery in animal model of type 2 diabetes it showed a significant decrease of blood glucose level (Jean et al. 2011). Successful intranasal vaccination of chitosan-DNA nanoparticles encoding pneumococcal surface antigen by Xu J et al. showed significant protection in mice against nasopharyngeal infection by *Streptococcus pneumonia* (Xu et al. 2011). Towards the end of the previous century when polyethylenimine (PEI) became popular as an efficient gene carrier with higher transfection efficiency, it superseded several old gene delivery vehicles including chitosan polymer. Therefore, chitosan nanoparticles needed an urgent attention to increase their transfection efficiency which leads to the development of several chemically modified chitosan derivatives like quaternized chitosan vectors, etc. Thanou et al. demonstrated spontaneous complex formation and increased transfection efficiency of DNA in COS-1 cells when complexed with trimethyl chitosan oligomer which was prepared by quaternization of chitosan molecule (Thanou et al. 2002). Another

approach for achieving success via chitosan-mediated delivery is to graft the chitosan nanoparticle surface with target-specific ligands. Selective binding of cyclic Arg-Gly-Asp (RGD) peptide with αvβ3 integrin, overexpressed on a number of tumor cells, can be utilized as a targeting strategy. RGD peptide fabricated chitosan nanoparticles have been widely used for tumor targeted gene delivery. Han HD et al. showed targeted gene silencing by utilizing RGD-labelled chitosan nanoparticles (Han et al. 2010).

In comparison to transfer of plasmid DNA, chitosan showed limited success about RNAi delivery. siRNA complexed with chitosan nanoparticles showed their successful delivery and inhibition of POSTN, FAK and Src family of genes in an animal cell model of ovarian cancer (Han et al. 2010; Kim et al. 2011). Yang J et al. reported induction of apoptosis in CaSki cervical carcinoma cells by delivery of chitosan bound human papilloma virus (HPV) 16 E7 siRNA (Yang et al. 2013). Cationic chitosan hydrogel is another type of colloidally stable nanosized, cross-linked polymer networks which also has important application in siRNA delivery. High water content, greater payloads, biocompatibility protection from nuclease of entrapped biomolecules including siRNA make hydrogels potent gene carrier (Ma et al. 2014). Chitosan-hydrogel loaded with transglutaminase (TG2) siRNA or docetaxel plus TG2 siRNA showed enhanced therapeutic efficacy mice bearing A375SM and MDA-MB231 tumors without any systemic toxicity (Han et al. 2011).

Over the past few years, attempts were made to further improve the delivery efficacy of chitosan-based nanoparticles. Chitosan nanoparticles have been used as biomaterial scaffold system or as a copolymer, in conjunction with other nanocarrier. PEI, as we discussed above, can act as efficient gene carrier, but due to high cytotoxicity, their application is often discouraged. Many pilot studies had demonstrated that a combination of chitosan and PEI (chitosan/PEI blend or Chitosan-graft-PEI copolymer) can retain the high transfection efficiency with significant reduction of cytotoxicity. In a recent study, H Lu et al. developed a chitosan/DNA nanocomplex grafted with low molecular weight PEI for osteoarthritis-targeted gene delivery into chondrocytes and synoviocytes (Lund et al. 2004). The same group developed a porous chitosan scaffold system which was shown to carry Hyaluronic Acid/Chitosan/Plasmid-DNA nanoparticles encoding TGF-β1 and controlled release of DNA into the transfected cells (Lu et al. 2011). Few current researches established the credibility of chitosan-DNA nanoparticles as promising vaccine delivery candidate. C Sawaengsak et al. developed intranasal chitosan-DNA vaccines that conferred protection in mice against influenza virus subtype H1N1 and H3N2 (Sawaengsak et al. 2014).

Derivatives of Dextran

Dextran is natural, complex, branched polysaccharide chain composed of glucose molecules where the straight chain consists of α-1,6 glycosidic linkages between glucose molecules and the branches tether with the main chain by α-1,3 linkages. DEAE-dextran as a cationic polymeric nanocarrier is one of the oldest techniques used for introducing nucleic acids into cultured mammalian cells (Jin et al. 2014). Cationic dextran hydroxyethyl methacrylate (dex-HEMA)-based nanogels are

shown to be potential siRNA carrier. Naeye et al. developed a PEGylated formulation of dex-HEMA which was shown to greater sustenance in systemic circulation and the encapsulated siRNA was demonstrated to efficiently inhibit EGFP expression in a HuH-7 EGFP cell line without any severe toxicity (Naeye et al. 2010). The advantages of this method are its relatively simple method of preparation, easier cell internalization etc. But, the present usage of dextran has been superseded because of other newly developed sophisticated nanocarrier system and cytotoxicity issues.

Cyclodextrins are a family of oligosaccharides where the single monomeric α-D-glucopyranoside units are cyclically linked via (α-1, 4) glycosidic linkage. Typical cyclodextrins are composed of 6–8 glucopyranoside units which are geometrically arranged in a shape of a toroid with one larger and one smaller opening (Jin et al. 2014). This unique shape gives them an extraordinary ability to form inclusion complexes with 'guest' molecules, suitably fitting in their hydrophobic chamber. Apart from efficient complexation ability, the application of CDs as the gene delivery vehicle is also favoured because of plenty of reasons which are as follows: (1) Cyclodextrin polymer, due to efficient packaging, can safely transport drugs at their target sites and thus, in turn, can facilitate their bioavailability, (2) low toxicity and absence of immune stimulation which promotes their biocompatibility, (3) enhanced resistance from degradation by serum nucleases, etc. (Davis and Brewster 2004).

The cyclodextrin delivery system was first developed to transfer plasmid DNA in 1999 (Gonzalez et al. 1999). Cyclodextrin polymer-based nanoparticle-mediated delivery of therapeutic nucleic acids stepped into the arena of clinical trial less than a decade after their introduction. Thereafter, a great length of research has been undertaken to improve cyclodextrin-based drug delivery chemistry and develop newer varieties of cyclodextrin-based novel vectors for gene delivery (Hwang et al. 2001; Pun et al. 2004). Cyclodextrin containing polycationic nanoparticles can self-assemble with siRNA to form colloidal particles of ~50 nm in diameter (Davis et al. 2004). Moreover, due to protection from external nucleases, siRNAs do not need any further chemical modification when complexed with cyclodextrin polymer. We already have discussed 'CALAA-01', the first targeted siRNA drug delivery system, manufactured by Calando Pharmaceuticals which was based on cyclodextrin polymer nanoparticle (Sharma et al. 2014). There is a variety of approaches imparting positive charges to cyclodextrin polymers which helps in efficient complexation with nucleic acids or oligonucleotides. J Li et al. synthesized cyclodextrin polymer via a step growth polymerization reaction between diamine-bearing cyclodextrin monomers and dimethyl suberimidate, yielding amidine functional group bearing oligomers (Li et al. 2004). These strong cationic amidine groups facilitate efficient nucleic acid condensation at lower N/P ratio. Cyclodextrin-based cationic polymers can also be prepared via 'click polymerization'. S Srinivasachari et al. demonstrated the potential for plasmid DNA delivery of high molecular weight linear polymers synthesized by 'click polymerization' of acetylated-diazido-β-cyclodextrin and α,ω-dipropargylated oligoethyleneimines (Srinivasachari and Reineke 2009; Nielsen et al. 2010).

Cyclodextrin polymers have been used for antisense oligonucleotide (AONs) delivery. For example, Chen et al. synthesized a pH-responsive acetylated cyclodextrin to deliver AONs targeted to Bcl-xL (an anti-apoptotic protein) in human lung adenocarcinoma cells (Chen et al. 2013). Although Cyclodextrins have been proved to mediate successful delivery of siRNA, these complexes require additional modifications with functional entities, such as targeting ligands, coating polymers to gain target specificity, higher stability and sustained efficacy in vivo. In order to overcome salt-induced agglomeration of cyclodextrin polymer-siRNA complex in vivo, adamantane-PEG (AD-PEG) has been incorporated into the system which resulted in improved delivery efficacy. Adamantane, being a hydrophobic molecule, formed a stable inclusion complex with the cyclodextrin core structure, and thus the nanoparticle system became non-covalently stabilized (Pun and Davis 2002; Park et al. 2006). PEG shielding provides necessary prevention of aggregation but significantly affects cellular uptake and thus silencing efficacy. In order to retain the stability as well as efficacy, adamantane-based chemistry was further extended to allow the conjugation of a targeting ligand, transferrin (Tf) to the free end of AD-PEG. This AD-PEG-Tf conjugate mediates multivalent binding to the Cyclodextrin71 Tf receptor (Bellocq et al. 2003). Shortly, thereafter, the first in vivo proof-of-concept experiments were performed with Tf conjugated Cyclodextrin polymer mediated delivery of siRNA targeting EWS/Fli1 fusion oncogene in a metastatic murine model of Ewing's sarcoma (Hu-Lieskovan et al. 2005). The significant antitumor effect shown in this work ultimately encouraged Calando Pharmaceuticals to undertake the development of cyclodextrin-based targeted siRNA drug 'CALAA-01'. In order to promote endosomal escape and sustained release of nucleic acids, the polymer termini are often end-protected by imidazole functional group (Mishra et al. 2006). Another very recent cyclodextrin-based targeted delivery of siRNA was shown by J C Evans et al. where they developed folate-targeted and poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE)-coated cyclodextrin-siRNA nanoparticle. This nanoparticle system showed enhanced uptake via prostate cancer specific antigen (PSMA) receptor (binding folate), abundantly expressed in PSMA (+) cancer cell lines VCaP and LNCaP, in comparison to untargeted controls. Overall, the study also highlights cyclodextrin-siRNA complex brought significant reduction in the level of target mRNA (Evans et al. 2016). In addition to this, cyclodextrin units are often covalently linked to various polycations such as PEI to enhance their transfection efficiency without eliciting any severe side effects. Li JM et al. demonstrated reversal of multidrug resistance in MCF-7/Adr cells by simultaneous delivery of Bcl2 siRNA and doxorubicin using a folate ligand-targeted PEI-capped hydroxypropyl-β-cyclodextrin nanocarrier. This work also showed how the PEI/cyclodextrin nanoformulations served as multifunctional copolymer nanocarrier for simultaneous gene and drug delivery (Li et al. 2015). Another such example of combinatorial therapy is simultaneous delivery of docetaxel and MMP-9 siRNA plasmid in cancer cells via a cyclodextrin derivative (Cyclodextrin-PLLD) composed of a β-cyclodextrin core and poly (l-lysine) dendron arms which significantly resulted in good transfection and subsequent reduction of MMP9 protein (Liu et al. 2016a). Despite a myriad

of research on the use of cyclodextrin and their derivatives as excipients for pharmaceutical purposes in the past few decades, the successful translation of this nanosystem to clinical reality remains a challenging as well as painstaking job. One of these limiting factors is the staggering cost of manufacturing the promising cyclodextrin-based delivery systems with clinical significance. More comprehensive and systemic evaluation and nanotoxicological studies are required newer cyclodextrin derivatives or cyclodextrin-containing polymers in order to obtain a much clearer clinical perspective of them.

Polyethyleneimine (PEI) is a polymer of repeating units of an amine group and two carbon aliphatic -CH_2CH_2 spacer. PEI is a class of extensively studied cationic polymers for gene delivery. The polymer comes in two forms, linear (lPEI) and branched (bPEI). The linear polymer contains secondary amines whereas the branched form has primary, secondary and tertiary amino groups (Yemul and Imae 2008). Due to the presence of closely spaced nitrogen atom along the polymer, PEI has a high charge density at lower or acidic pH values. This property facilitates efficient DNA condensation via electrostatic binding and endosomal escape via proton buffering capacity. That's why PEI is known as the gold standard for plasmid DNA delivery (Dunlap et al. 1997). PEI was the second cationic polymer-based gene transfection reagent next to poly-L-lysine (PLL). A wide variety of cells has been tested for PEI-mediated transfection. Boussif et al. reported for the first time the high transfection ability of PEI, both in vivo and in vitro (Boussif et al. 1995). However, the PEI and its numerous derivatives though have received significant attention due to their high transfection efficiency, but they also impose serious threat of cytotoxicity which impedes their development and wider acceptance as therapeutic nucleic acid carriers in clinical settings (Kafil and Omidi 2011). Because of this toxicity issue, its application for stable systemic siRNA transfection is rather less popular.

In recent years, in-depth investigations of cellular processes related to PEI-mediated transfections have been conducted in order to address the dilemma between transfection efficiency and adverse side effects of PEI. Soon after the discovery of PEI's extraordinary ability to promote gene transfection in vitro and in vivo, continuous efforts have been made for better understanding of its structural property and related chemistry. Extensive studies revealed that the transfection efficiency and cytotoxicity of PEI majorly depend on its polymer: plasmid ratio, molecular weight and degree of branching (Jin et al. 2014). It has been observed that these PEI properties can be smartly controlled and manipulated in order to design more biocompatible polymer. For example, the fine-tuning of the ratio of polymeric nitrogen to DNA phosphates (N/P ratio) can significantly affect the transfection efficiency, aggregate formation and cytotoxicity. The polymer/DNA complex with N/P ratio a little higher than 3 contains excess free PEI, sufficient to contribute the endosomal escape (Boeckle et al. 2004; Nimesh et al. 2007; Vu et al. 2012). But very high N/P ratio or an overall positive charge can stimulate complement system activation whereas reducing the positive charge up to a threshold limit so that DNA complexation does not get impaired, complement activation gets reduced and trans-

fection associated cell death also becomes less (Plank et al. 1996; Suk et al. 2016; Ogris et al. 1999). Another important criterion which significantly determines the transfection efficiency of PEI/plasmid complex is the molecular weight. PEI with high molecular weight (PEI_{HMW}) has high transfection efficiency but lacks biodegradability which, in turn, contributes to acute cytotoxicity. On the other hand, in comparison to high molecular weight PEI, use of low molecular weight PEI (PEI_{LMW}) displays not only negligible cytotoxicity but also reduced transfection efficiency. Talking about branching, PEI with more branched structure can condense to a greater extent than do their linear counterpart (Jin et al. 2014).

Studying the physicochemical properties of PEI makes us understand that designing of PEI-based vectors with high transfection efficiency and low toxicity is significantly a challenging job. In order to achieve a balance between these two relatively incompatible factors, transfection efficacy and cytotoxicity, several approaches have been adopted. For PEI_{HMW}, cross-linked PEI containing reducible disulphide bonds have been used to lower the cytotoxicity while maintaining high transfection efficiency of plasmid DNA (Breunig et al. 2008; Liu et al. 2010a; Breunig et al. 2007). Cytotoxicity issues can also be resolved by the introduction of hydrophobic functional groups like polycaprolactone (PCL) (Endres et al. 2011), lipids (Bahadur et al. 2011; Liu et al. 2010b) into the PEI-polymeric vector. Osukee et al. introduced alkylcarboxyl group to branched PEI to impart hydrophobicity, and the resulting polymer showed significant efficient intracellular internalization, reduction of cytotoxicity and improved silencing efficacy (Oskuee et al. 2010). Block copolymers of PEI like PEI-PEG (Mao et al. 2006) and PEI-g-PCL-block-PEG-folate (Liu et al. 2016b) have been widely used as efficient gene carrier with lower systemic toxicity. Protein binding to PEI/DNA surface during systemic circulation (which ultimately leads to the formation of large aggregates) can be prevented by PEGylation of PEI surface (Suk et al. 2016; Ogris et al. 1999).

Other than working alone as a gene carrier, PEI polymers are most often used as surface coating polymers, cross-linkers and structural moieties of other block copolymers with excellent functionalities. Lee Y et al. synthesized catechol-grafted PEI-coated gold nanoparticles for siRNA delivery with tunable sizes and surface charges which showed great electrostatic binding affinity to siRNA and excellent gene silencing activity in cancer cells (Lee et al. 2011). Plenty of reports are presently in the archive where inorganic material-based siRNA nanocarriers like magnetic nanoparticles, mesoporous silica, carbon nanotubes and graphene oxide are prepared with a positively charged PEI polymer layer on their surface which ultimately helps in electrostatically binding with negatively charged siRNA and stabilizing the nanoparticle. To magnetically deliver siRNA, Park et al. have developed clustered magnetic nanocrytals crosslinked with bPEI (Park et al. 2011). In an attempt to design a multifunctional targeted PEI-based gene carrier, different ligands, for example, antibody (e.g. anti-HER2 antibody (trastuzumab, Herceptin®)) (Chiu et al. 2004), folate (Teo et al. 2015), transferrin (Xie et al. 2016) have been coupled to the PEI polymer surfaces.

PEI has versatile application in the context of nanoparticle-mediated gene delivery. With the increased understanding of polymer chemistry and surface engineer-

ing of nanoparticles newer PEI derivatives with improved benefit-to-risk ratio are being developed. These new-age derivatives still need lot more intense investigations before being translated into clinical applications.

Poly (2-N, N-Dimethylaminoethyl Methacrylate) (pDMAEMA) is a member of weak cationic polyelectrolyte prepared from monomeric DMAEMA, mainly by controlled radical polymerizations. In 1996, Hennink group for the first time demonstrated that pDMAEMA can be utilized as a gene delivery system and can achieve comparable transfection efficiency of PEI- and lipid-based formulation (Cherng et al. 1996). Since then, researcher has studied several properties of this polymer in connection with their suitability as efficient transfection agent including molecular weight, size, polymer/plasmid ratio and other gene delivery parameters. For example, at low (polymer/plasmid) ratios, the polyplexes usually showed negative ζ potential and relatively larger size, both of which are unfavourable for intracellular internalization. At (polymer/plasmid) ratio above 3:1 (w/w), the polymer formulations are favourably small in size and have positive ζ potential (Rezvani Amin et al. 2013).

Like PEI, these properties of pDMAEMA also require fine modulation in order to achieve safe and significant gene transfection. Few strategies were adopted in order to circumvent the lethal cytotoxicity albeit maintaining the gene transfer and expression efficiency of pDMAEMA. Design of copolymer is an effective alternative route to achieve the necessary optimization of efficacy vs. toxicity ratio. Thus, PEGylation of pDMAEMA surface via formation of block copolymers can reduce the toxicity and enhance the duration of circulation (Mathew et al. 2012; Lin et al. 2008; Georgiou et al. 2006). But, introduction of PEG is accompanied by the compromise of transfection efficiency. Therefore, pH-responsive functional linkers like acetal, hydrazone are considered for coupling PEG to the polycation. Under acidic environment these linker molecules undergo acid hydrolysis that ultimately results in deshielding polymer core (Mathew et al. 2012; Lin et al. 2008; Georgiou et al. 2006; Park et al. 2010). Besides this, bringing a hydrophobic moiety like polycaprolactone between pDMAEMA and PEG block is often favoured (Yue et al. 2010). In order to retain the transfection efficiency, some statistical copolymers of pDMAEMA with hydrophobic monomers, such as methyl methacrylate (MMA) or hydrophilic, and amphiphilic monomers like N-vinylpyrrolidone (NVP) and ethylene glycol methacrylate (EGMA), respectively, have been evaluated. Studies revealed that the introduction of hydrophobic monomers was suggested to be less attractive approach in order to attain an optimized ratio of efficacy vs. toxicity whereas copolymer with NVP showed reduced toxicity and enhanced transfection efficiency (van de Wetering et al. 1998). A systemic study revealed that along with size and molecular weight other structural aspects including the degree of branching, number of arms and shape also influence the gene delivery process. Several groups found better transfection results when branched structures are used instead of linear ones (Synatschke et al. 2011; Agarwal et al. 2012). It was found that star-shaped pDMAEMA with multiple arms results in better transfection efficiency combined with low cytotoxicity (Georgiou et al. 2006).

In recent years, few research attempts have been made to increase the biodegradability of pDMAEMA polymer. In general degradable cationic polymers have labile linkages such as intracellularly reducible disulphide bonds, hydrolytically cleavable functional groups like esters, acetals, etc. (Luten et al. 2008) You et al. demonstrated for the first time that pDMAEMA copolymers with reducible disulphide linkages synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization show comparable gene delivery efficacy and cytotoxicity, like homo-pDMAEMA (You et al. 2007). Very recently, Yang et al. synthesized a novel star-shaped reducible gene vector of DMAEMA via atom transfer radical polymerization (ATRP) from a polyhedral oligomeric silsesquioxane (POSS) macroinitiator where the biocompatible POSS core are linked to eight disulphide-linked pDMAEMA arms [POSS-(SS-PDMAEMA)$_8$] (Yang et al. 2014). The copolymer showed excellent transfection efficiency and reduced cytotoxicity. Disulphide-linked biodegradable systems are often used for surface functionalization of other nanoparticles carrying therapeutic nucleic acid or RNAi machinery. For example, D Lin et al. developed cleavable pDMAEMA functionalized mesoporous silica nanoparticles for efficient siRNA delivery both in vitro and in vivo without any noticeable toxic side effects (Lin et al. 2013).

Though it has been found successful by many research groups, designing of an ideal and clinically reproducible pDMAEMA-based gene delivery platform is a multidisciplinary problem which requires more inclusive knowledge about polymer chemistry and precise systemic analysis of compositions, architectures and functionalities of its various derivatives.

Dendrimers are regular, spherical, highly symmetric and highly branched macromolecules. Their unique physicochemical properties like well-defined structure, homogeneity, hydrophilicity, highly functionalized terminal surface, tunable molecular weight and sizes make them an attractive option for gene delivery vector. Different polycationic dendrimers, poly(amidoamine) (PAMAM) and poly(propylenimine) (PPI)-based dendrimers, have been extensively characterized and studied.

Polycationic PAMAM dendrimers have their surfaces decorated with primary amine groups which facilitate nucleic acid binding, nanoparticle formation, intracellular internalization and tertiary amine groups facing inwards which promotes endosomal escape and proper release of nucleic acids into the cytoplasm (Singha et al. 2011). The whole structure originates from a core molecule and then is grown with branches of repeated units via stepwise polymerization process (Jin et al. 2014). For each addition of each new layer (or, increase in generation number) the molecular weight increases. With increase in generation number, the density of the surface branching units also increases which confer structural diversity to the shape of the whole complex. Generally, high generation dendrimers evolve with a hydrophobic space which helps in encapsulation of various pharmaceutically important compounds (Jin et al. 2014). The amine group rich surface can further be functionalized with targeting ligands, antibodies, contrast agents which promotes drug delivery efficacy (Luten et al. 2008; You et al. 2007). Besides these properties, their hyper branched and monodispersive nature also makes them suitable for gene deliv-

ery (Yang et al. 2014). In addition to these, dendrimers are comparatively smaller than other nanocarriers (e.g. liposomes, NPs etc.) due to which they can easily be conjugated with other standard nanocarriers (i.e. mesoporous silica NPs, carbon-based NPs) (Lin et al. 2013; Singha et al. 2011).

PAMAM based cationic nanoparticles. Dendrimer toxicity is primarily dependent on three factors, namely, i) generation number, ii) surface charge and iii) concentration (Yang et al. 2014; Saraswathy et al. 2015). High generation PAMAM (G_4-G_8) showed high transfection efficiency as well as higher toxicity, mainly because of non-biodegradability whereas low generation PAMAM (G_0-G_3) have poor transfection efficiency and low cytotoxicity (Brito et al. 2014; Saraswathy et al. 2015). High generation PAMAM, due to high charge density dendrimer-membrane interaction results in loss of membrane integrity which finally leads to leakage of intracellular components and thus acute toxicity induced cell death (Saraswathy et al. 2015; Wu et al. 2013b). Thiagarajan et al. evaluated that the maximum tolerated dose of cationic dendrimers is ~10 times lower than anionic dendrimers (Thiagarajan et al. 2013). Several approaches have been adopted in order to counteract the cytotoxicity issues while maintaining high degree of transfection efficiency. One interesting method is to use flexible dendrimers. Zhou et al. developed a PAMAM dendrimer-based siRNA delivery strategy where triethanolamine was used as core entity and the first-generation branching started ten successive bonds away from central nitrogen atom resulting in highly flexible dendrimer and thus increases siRNA delivery and gene silencing was observed (Zhou et al. 2006). Liu et al. delivered heat-shock protein 27 (Hsp27) siRNA into human prostate cancer (PC-3) cells via a structurally flexible PAMAM G_7 dendrimers having triethanolamine core. This work showed pronounced gene silencing and induced caspase-dependent apoptosis (Liu et al. 2009). To counteract the cytotoxicity, the Minko group developed siRNA nanocarrier containing PAMAM dendrimers with modified surface end-group functionalities, PAMAM-OH and PAMAM-NHAc dendrimers. They further modified the structure by the introduction of internal tertiary nitrogen which was quaternized. Thus, the internally quaternized and surface neutral dendrimers showed efficient electrostatic binding with siRNA and better protection of siRNAs from external nucleases due to the formation of highly organized and densely packed nanoparticles. Moreover, the neutral surface of the quaternized dendrimers helps in overcoming serum induced protein aggregation problem. However, only PAMAM-NHAc dendrimers showed targeted intracellular delivery of Bcl-2 siRNA in A2780 human ovarian cancer cells (Patil et al. 2008). Later the same group achieved the targeted delivery of Bcl-2 siRNA with PAMAM-OH dendrimers, and substantial silencing was observed when the nanoparticles were conjugated with a synthetic analog of luteinizing hormone-releasing hormone (LHRH) targeting peptide (Patil et al. 2009). In subsequent years, several other targeting ligands like cyclic RGD peptide, folate and antibody in conjunction with PAMAM dendrimer have also been used (Saraswathy et al. 2015; Xu et al. 2016; Ma et al. 2015). Other modifications adopted to augment the gene delivery efficacy are as follows: the introduction of L-arginine on the dendrimer surface (Choi et al. 2004b), PEGylation (Yuan et al. 2010; Luong et al. 2016) and designing of block copolymer

like triblock PAMAM-PEG-PLL nanocarrier where PLL being a cationic polymer further facilitates siRNA binding and PEG acts both as a linker between PLL and PAMAM and stabilizer (Patil et al. 2011). As dendrimers can improve gene delivery efficiency, many scientists have expressed genuine interests in dendrimer-based nanohybrid gene carrier systems (Kesharwani et al. 2018). PAMAM modified magnetic iron oxide NPs loaded with DNA have shown high transfection efficiency. Magnetofection results in rapid accumulation of the ternary NPs/DNA/PEI ternary magnetoplexes into the targeted COS-7 cells (Liu et al. 2011a).

PAMAM, due to their high compaction quality and ability to protect encapsulated drugs from external environment, often are favoured as a material to functionalize or shield inorganic nanoparticles carrying siRNA. For example, Xiao et al. reported the delivery of plasmid DNA encoding enhanced green fluorescent protein (EGFP) via a folate-targeted generation 5 (G_5) PAMAM entrapped gold nanoparticle (AuNP) (Xiao et al. 2013).

Recently, Qiu J et al. reported the use of β-cyclodextrin grafted G_5 PAMAM to entrap AuNP and their ability to deliver plasmid DNAs encoding luciferase and enhanced green fluorescent protein into 293 T cells efficiently without arousing any cytotoxicity (Qiu et al. 2016). Liu X et al. developed a hybrid PAMAM and oleic acid functionalized graphene-based non-viral nanosized gene delivery vector which shows good dispersity and stability in aqueous solution and high gene transfection efficiency (Liu et al. 2014). In another recent study, Pourianazar et al. delivered CpG ODNs in MDA-MB231 and SKBR3 tumor cells by loading them into PAMAM dendrimers coating iron oxide magnetic core (Taghavi Pourianazar and Gunduz 2016).

Besides PAMAM, poly(propylenimine) (PPI)-based dendrimers have also been utilized as non-viral vectors. PPI generally consists of two types of nitrogen atoms, primary amine nitrogen and tertiary amine nitrogen. Like PAMAM, PPI dendrimers have also been subjected to various surface engineering approaches in order to increase their gene delivery efficacy and reduce cytotoxicity. Taratula et al. formulated PPI dendrimers /siRNA nanocomplex and modified it with dithiol cross-linker molecules followed by PEG coating. The nanocomplex was further modified by conjugating an analog of LHRH and PEG in order to specifically direct it towards the cancer cells (Taratula et al. 2009, 2011). Very recently, Tietze et al. developed a polyplex nanocarrier system for targeted delivery of siRNA based on maltose-modified poly(propyleneimine)-dendrimers (mal-PPI) bioconjugated to monobiotinylated anti-epidermal growth factor receptor variant III single chain fragment variable (EGFRvIII-ScFv) fused with a biotinylation acceptor. This dendrimer system reports excellent cellular uptake of siRNA by receptor mediated endocytosis (Tietze et al. 2017). High transfection efficiency with low generation PPI dendrimers was achieved by using gold nanoparticles (Au NPs) as a ‘labile catalytic’ packaging agent encapsulating siRNA into discrete nanoparticles. The gene silencing efficiency by this method is superior even in comparison to high generation dendrimers (Chen et al. 2010b).

Hybrid Nanoparticles

Different nanoparticle systems discussed here have drawn significant attention because of the versatile range of advantages they offer. Hybrid nanoparticle, a rapidly emerging platform of drug delivery method, is an integrated system in which different compatible nanoparticles are combined in order to use the potentials of each of these constituent nanoparticles to their fullest extent. This group of superior system of nanoparticles often is seen to mitigate the toxic effects of constituent nanoparticles, if any, when they are used alone. We already have discussed how a coating of cationic lipid improved the PEI-mediated transfection efficiency of DNA and concomitantly reduced cytotoxicity. Different types of hybrid nanoparticle systems are currently being studied, for example, lipid-polymer hybrid nanoparticles (LPHNs), polymer-inorganic material hybrid nanoparticles etc. Among different types of nanohybrid structures, lipid-polymer hybrid nanoparticles (LPHNs) have received significant attention and here, we will discuss about different types of LPHNs.

Lipid-Polymer Hybrid Nanoparticles (LPHNs)

Among various cationic lipid-based non-viral gene carriers, liposomes have been most extensively studied. Their significant popularity as ideal gene delivery tool mainly originated because of their high encapsulation efficiency, high transfection efficiency with low level of cytotoxicity and ease of preparation. Despite these advantages, liposomes suffer some drawbacks like fragileness resulting in premature drug leakage and large batch to batch variation etc. Therefore, researchers have sought an ideal alternative of this nanocarrier system which ultimately leads to the development of cationic polymer-based drug delivery. With the progress of polymer-based gene transfer methods, it was observed that the success of this technology has also become burdened with severe cytotoxicity issues. While use of naturally available biopolymers can alleviate the toxicity to some extent but they do not offer a wider scope of surface functionalization as their synthetic counterpart which in turn, has impeded their broad range of applications. Therefore, a great length of research efforts is currently being oriented on the reduction of polymer toxicity with the concomitant maintenance of their high transfection efficacy. Simultaneously, an alternative branch of research has been focusing on the amalgamation of several complementary advantages of both the cationic lipids and polymers which gave birth of the lipid-polymer hybrid nanoparticles (LPHNs).

LPHNs, a new-generation therapeutic delivery vehicle, are core-shell nanohybrid system, comprising a polymer core with a lipid/lipid-PEG shells. As discussed above, they exhibit both the characteristics of polymeric nanoparticles as well as liposomes (Mandal et al. 2013; Hadinoto et al. 2013). The polymer core provides structural integrity and stability which, in turn, confers the system a controlled drug release character whereas the lipid shell creates a characteristic hydrophobic, protective environment at the interface with the polymer core by preventing inflow of water (Mandal et al. 2013; Hadinoto et al. 2013; Chan et al. 2009). Moreover, lipid shell, either alone or in combination with another outer PEGylated layer, also helps

to increase biocompatibility and bioavailability (Chan et al. 2009). Similar to other nanoparticle system an additional layer of PEG improves colloidal stability reduces immunogenicity. PEGylation also offers an additional advantage of surface functionalization by modification of end-group chemistry (Salvador-Morales et al. 2009). Different targeting ligands like folate (Zhao et al. 2012), transferrin (Zheng et al. 2010) and A10 aptamers (Zhang et al. 2008) can be fused with the LPHNs for specific tropism of chemotherapeutic drugs.

Conventionally, LPHNs were prepared by a two-step method involving introduction of introducing preformed lipid vesicles into preformed polymeric core. Due to systemic and manufacturing difficulties, alternatively a single-step strategy has been developed which relies on the fabrication process of either nanoprecipitation or emulsion-solvent evaporation (ESE) in combination with the simultaneous self-assembly of the lipid and polymer (Mandal et al. 2013; Hadinoto et al. 2013). The physicochemical properties of LPHNs including structure, size homogeneity and stability as well as loading and encapsulation efficiency depend on interaction between lipid and polymer layer which on the other hand is determined by the method of preparation. It was shown that monodispersity of LPHNs prepared by two-step method depended on the charge and size uniformity of the lipid vesicles (Mandal et al. 2013; Hadinoto et al. 2013; Troutier et al. 2005). The colloidal stability of this type of LPHN was shown to depend on the chain length of lipid moiety, vesicle to polymer ratio etc. (Troutier et al. 2005). To the best of our knowledge, very few studies showed the effects of various formulation parameters on the loading and encapsulation efficiency of LHNPs prepared by two-step method as because, for the LPHNs prepared by the two-step method, these formulation parameters are no different from those investigated exhaustively for the polymeric nanoparticles in the past; hence, similar studies were not repeated for the preparation of LPHNs from the same polymeric nanoparticles. Here we will mainly focus on the one-step preparation method which, as discussed above, is technically a better method.

One of the important formulation parameters in single-step preparation method, significantly involved in determining several features of LHNPs, is lipid to polymer mass ratio (L/P ratio). Different groups showed the influence of L/P ratio on the spatial configuration of lipids, drug encapsulation efficiency, loading and release kinetics of the particle assembly (Mandal et al. 2013; Hadinoto et al. 2013). Higher L/P ratios (above the critical micelle concentration) result in the formation of multilamellar lipid coatings or free liposomes in addition to the LPHNs (Bershteyn et al. 2008), whereas lower L/P ratios led to LPHN aggregation due to insufficient lipid coating. The formation of free liposomes often results in loss of drug encapsulations as they are washed away during purification. Therefore, optimization of L/P ratio or the amount of lipid required to uniformly coat the polymer core surface is of utmost importance. It was shown both by Zhang et al. and Chan et al. that the L/P ratio of ~ 15% (w/w) in the reaction was optimum to sufficiently cover the surface of the PLGA core in order to manufacture stable LPHNs in the size range of ~ 60–80 nm by nanoprecipitation method (Chan et al. 2009; Zhang et al. 2008). The report by Zhang et al. also suggested that at that optimal L/P ratio in terms of size (15%), the LPHNs exhibited higher drug encapsulation efficiency of the anticancer

drug docetaxel in comparison to non-hybrid PLGA and PEGylated-PLGA nanoparticles (Zhang et al. 2008). On the other hand, Cheow et al. studied the effects of L/P ratio of LPHNs prepared from PLGA (polymer core), phosphatidylcholine from soya bean lecithin (lipid layer) and TPGS (surfactant) by ESE. An L/P ratio of 30% (w/w) was found to be optimum for maximum LPHNs yield, and deviation from this ratio caused particle aggregation and size reduction with lower yields (Cheow and Hadinoto 2011). Liu et al. prepared LPHNs using PLGA and 1, 2-dilauroyl-sn-glycero-3- phosphocholine (DLPC) precursors, which also showed that higher L/P ratios resulted in smaller LPNs (Liu et al. 2010c). Recently, Bose et al. studied the effect of the concentration of cationic lipids on the morphology and gene transfection efficiency of LPHN assembly prepared from positively charged cationic lipid (DOTAP) with a protamine layer (condensing agent) forming the shell and an inner spherical PLGA core (Bose et al. 2015). They showed that with the increase of lipid concentration, the particle size decreases and DNA incorporation and transfection efficiency increase. Additionally, presence of protamine further helps in DNA condensation.

The application of LPHNs for gene delivery has flourished in the last few years. A famous study by Zhong et al. evaluated the transfection efficiency of plasmid DNA encoding luciferase (pLuc) gene delivered via three different formulations of LPHNs. The polymer and lipid precursors of the LPHNs are PLGA and DOTAP or DC-Chol, respectively. The three different formulations are distinct from each other in terms of the location of the packaged DNA: (1) 'OUT' method (as referred by Zhong et al.), DNA adsorbed onto the cationic lipid shell of the LPNs; (2) 'IN' method, DNA is packaged into the aqueous core of the LPHNs; and (3) 'BOTH' method, a combination of 'OUT' and 'IN'. All of them showed adequate intracellular uptake, but the 'OUT' method showed large initial uptake during a 4-week incubation followed by steep decline whereas the other two methods exhibited sustained pLuc activity (Zhong et al. 2010). Therefore, the 'OUT' method is suitable for prompt and strong gene delivery. On the other hand, the sustained gene expression activity for 'IN' and 'BOTH' was best seen in LPNs with DOTAP, whereas the LPHNs with DC-Chol exhibited best compatibility in 'OUT' method. Thus, it became clear that lipid formulation can be modulated in the LPHN/DNA complex according to our need. Li et al. later formulated an LPHN by 'OUT' method comprising PEI core and the lipid shell of PC/triolein/DSPE-PEG which was shown to successfully transfect plasmid DNA encoding green fluorescent protein (pEGFP-N2) into both the HEK 293 cells and the MDA-MB-231 breast cancer cells, and they also reported that the transfection efficiency is higher than the commercially available lipofectamine (Li et al. 2010).

LPHNs were also shown to successfully deliver mRNA molecule. Su et al. designed a LPN/mRNA complex by electrostatic adsorption of mRNA to the preformed LPHNs composed of PBAE core and DOPC/DOTAP/DSPE-PEG lipid shell. The resulting nanocomplex showed successful delivery of mRNA to the dendritic cell without arousing any toxicity (Su et al. 2011).

Besides DNA and mRNA, LPHNs were explored for efficient delivery of siRNA. Hasan et al. employed a soft lithography particle moulding technique

termed as 'Particle Replication in Non-Wetting Templates (PRINT)' to encapsulate siRNA into PLGA polymer core coated with DOTAP and DOPE as lipid shell. This nanoparticle system showed effective gene silencing in HeLa cells (Hasan et al. 2012). Shi et al. developed a neutral surface charged hybrid nanostructure referred as 'differentially charged hollow core/shell lipid-polymer-lipid hybrid nanoparticles' composed of cationic lipid, PLGA layer and a neutral lipid layer with protruding PEG chains. This hybrid nanostructure showed highly effective and sustained release of siRNA and silenced in vivo luciferase gene expression (Shi et al. 2011).

Virus-Like Particles

We already have discussed how several drawbacks of viral vectors including immunogenicity, carcinogenesis, limited loading of cargo molecules and non-specificity have influenced the origin and development of non-viral vector-based gene therapy. Most of the present non-viral vector mediated delivery of therapeutic nucleic acids involves lipid and polymer-based nanoparticles. Despite several studies over the past two decades, clinical approval of these NP-based drugs has been impeded mainly by the lack of proper optimization of their efficacy in comparison to the adverse systemic response. Therefore, newer derivatives of conventional nanomaterials with better surface engineering and novel drug delivery platforms are being studied in recent years. Among these alternative drug carrier candidates, virus-like particles (VLPs) have shown significant promise.

VLPs are biological nanoconstructs, engineered from viral capsids but devoid of viral genome and thus are not able to cause infections (Rohovie et al. 2017). VLPs have emerged as a promising class of targeted delivery vehicles which have the potential to address several conventional technical challenges. Several groups have already shown that VLPs can entrap and deliver bioactive, therapeutic cargos including chemotherapeutic drugs, siRNA, RNA aptamers, proteins and peptides. VLPs are more uniform than polymer NPs and highly stable than liposomes. VLPs are much less cytotoxic than inorganic NPs and are easily expressed in bacteria (Rohovie et al. 2017). Since the first demonstrated use of hepatitis B virus core protein (HBc) VLPs as an antigen carrier in 1987 (Clarke et al. 1987), at least capsid proteins from 35 different virus families have been used to manufacture approximately 110 VLP vaccine candidates (Zeltins 2013). However, similar to other NPs, using of VLPs is also burdened with some limitations. For example, RES mediated clearance of VLPs from systemic circulation, even after PEGylation, is a major concern. In addition to this, stability is an issue. Moreover, it has been observed that making polymeric nanoparticles ellipsoidal in shape would favour their extravasation from the blood vessel to the targeted tissue but reengineering of icosahedral VLPs into ellipsoidal ones is not easily feasible (Rohovie et al. 2017). Therefore, a number of strategies such as surface functionalization with active ligands have been adopted to counter these challenges. Here, we will discuss the design considerations which involve cellular targeting, cargo loading and delivery and related biomedical

applications of the most extensively developed VLPs, more specifically for therapeutic delivery of nucleic acid.

Engineering of Artificial Viruses: Design Consideration in Developing VLPs

The concept of VLPs initially started to garner interests because of their wide range of applications, firstly as vaccine delivery materials with a high level of efficacy and safety. VLPs, owing to the high density of epitopes on their surfaces, can elicit humoral and cellular immunity effectively similar to the native pathogens but at the same time do not possess viral genetic materials which confer an added benefit of no infection (Liu et al. 2016a). A vast number of reports are present where VLPs have been used as a versatile platform for antigen presentation. Apart from delivery of therapeutic and prophylactic vaccines, recently VLPs have emerged as suitable delivery vehicles for small molecules (e.g. porphyrin), chemotherapeutic drugs (e.g. taxol and doxorubicin), fluorescent probes (e.g. fluorescein) siRNA, antisense oligonucleotides, RNA aptamers, proteins and peptides (Yemul and Imae 2008; Dunlap et al. 1997; Kafil and Omidi 2011; Boeckle et al. 2004; Nimesh et al. 2007; Vu et al. 2012; Plank et al. 1996; Suk et al. 2016; Ogris et al. 1999; Breunig et al. 2008). Different VLPs which have been elaborately studied and have been discussed here are hepatitis B virus core (HBVc), RNA bacteriophage MS2, Qβ, P22, Cowpea chlorotic mottle virus-like particles (CCMV), etc. Although VLPs can spontaneously self-assemble into a highly organized spatial nanosized conformation under appropriate condition, they also require a significant amount of internal and surface engineering for efficient packaging, retention and controlled release of drugs at targeted sites.

Surface Modifications

As VLPs are composed of proteins only, for many VLPs, peptide or amino acid sequences of the coat proteins can be modified to be presented on either the interior or exterior surfaces. On the other hand, in order to design multifunctional VLPs, another common approach is to fuse different chemical groups or ligands by covalent or non-covalent means on the surface of the particle. Similar to other NPs, these ligands serve different purposes to VLPs like avoiding immune detection, targeting specific cells, enhancing stability, bridging some other chemical moieties, detection etc. All this modification firstly starts with reactions which involve amino acids of surface coat. Here we will mainly discuss how different the reactive amino acid-based modifications are used to decorate VLP surface with different chemical moieties like cell-penetrating peptides (Anand et al. 2015; Wu et al. 2012), fluorescent probes (Kang et al. 2008), PEG molecules (Comellas-Aragones et al. 2009; Patel and Swartz 2011), targeting ligands (e.g., folic acid, transferrin, antibody fragments) (Destito et al. 2007; Galaway and Stockley 2013; Peyret et al. 2015), etc. Later a separate section (Cellular targeting and delivery) has been dedicated to discussing how and what targeting ligands are used specifically in association with VLPs for achieving higher therapeutic efficacy.

Cysteine amino acids either presented naturally or by mutation are often used to form reducible and biodegradable disulphide bonds with other sulphahydryl group containing ligands (Anand et al. 2015; Chatterji et al. 2004a, 2004b; Pomwised

et al. 2016;). Maleimide derivative compounds form thioether linkages with cysteine residues. This attachment chemistry has been reported to conjugate heterologous peptides, fluorescent detection probes etc. as mentioned before. Lysine amino acids, via n-hydroxysuccinimide (NHS) ester reactions, allow the attachment of transferrin on MS2 which was shown to permit the VLP to immigrate the blood-brain barrier (Galaway and Stockley 2013). Copper (I)-catalysed azide-alkyne cycloaddition ('click reaction') is a widely used reaction by which heterologous proteins, antibody fragments, nucleic acids, PEG and RGD peptide are covalently conjugated to azidohomoalanine (AHA), a non-natural methionine amino acid analog frequently incorporated in HBVc, MS2, Qβ and CCMV VLPs (Anand et al. 2015; Wu et al. 2012; Pan et al. 2012; Gillitzer et al. 2006; Brunel et al. 2010). An *Escherichia coli*-based cell-free protein synthesis (CFPS) system has been successfully developed for the incorporation of non-natural amino acids onto the outer surface (Carlson et al. 2012). Besides the direct amino acid modification, the gene for the desired surface ligand is often conjugated to the gene encoding the coat protein of the VLPs (Servid et al. 2013).

Cargo Loading

Surface modification not only serves decorating the VLP surface but also helps in cargo loading. We already have discussed that different types of cargo molecules like small molecules, chemotherapeutic drugs, fluorescent probes (fluorescein), nucleic acids, aptamers, peptides or proteins and other nanoparticles are loaded into VLPs. This is basically accomplished by conjugating with interior cysteines, lysines and the stem-loop RNA hairpin secondary structure required for VLP self-assembly. Cargos loading by covalent methods apply the same chemistries of reactive amino acids as discussed above and, in some cases, recombinant fusion with primary amino acid sequence is also employed. MS2 VLPs have been reported to be specifically suitable for encapsidating RNA efficiently better than other cargos. A short stretch of stem-loop RNA hairpin, typically a part of their genome, can be extended to incorporate mRNA, siRNA, miRNA, antisense oligonucleotides, etc. For example, Wu M et al. reported MS2 RNA bacteriophage-mediated delivery of antisense oligonucleotides targeting p120 mRNA in myelogenous leukemia cells (Wu et al. 2005). In this work, antisense oligonucleotides have been synthesized as covalent extension of a 19 nt long stem-loop translational repressor/assembly initiation signal (TR) mRNA. Storni T et al. showed induction of cytotoxic T cell responses without any systemic side effects by nonmethylated CG motifs (CpGs) encapsidated into virus-like particles (VLPs) derived from the hepatitis B core Ag or the bacteriophage Qβ (Storni et al. 2004). Pan Y et al. described inhibition of autoantibody production by MS2 VLP-based delivery of miR-146a (Pan et al. 2012). Very recently, Hoffmann et al. developed polyoma JC virus-derived VLPs to deliver siRNAs targeting the receptor activator for nuclear factor-kappaB ligand (RANKL) in osteoblast cells in vivo (Hoffmann et al. 2016). Another interesting report published by Ashley et al. showed the use of a single formulation of bacteriophage MS2 VLPs for selective delivery of target specific peptide conjugated Qdot® 585 ITK™ amino(PEG) quantum dot nanoparticles, chemotherapeutic drugs (doxorubicin,

cisplatin, 5-flurouracil), siRNA and ricin toxin A-chain to human hepatocellular carcinoma (HCC) (Ashley et al. 2011). Other examples of nanoparticle loading into VLPs are HBVc and CPMV VLPs which have been loaded with iron oxide nanoparticles by conjugation to coat proteins or through passive encapsidation (Shen et al. 2015; Aljabali et al. 2010). HBVc, P22 and CCMV VLPs have been reported to be suitable for RNA loading by electrostatic interaction with coat proteins. Encapsulating and retaining of nucleic acid into VLPs is comparatively easier than other small molecules because capsids have evolved to carry similar molecules, viral genome. We already have reported that VLPs have been useful for protein loading and their delivery also. Few considerations are taken into account prior peptide or protein loading and the foremost of which is that the loaded peptide or protein must be able to fold itself into an active form while not interfering with the packaging of nucleic acid or other cargos and the folding of VLP subunits. On the other hand, the protein must show its effect while remain conjugated to the coat protein or the nucleic acid (Rohovie et al. 2017). Peptide or protein loading is currently accomplished by several approaches such as covalent conjugation to the amino acid sequence of coat proteins, direct fusion with the genome, electrostatically bounded to the coat or cargo molecules and passive loading (Rohovie et al. 2017). MS2 and Qβ VLPs were reported to encapsidate peptides and proteins by conjugating them to the RNA containing the stem-loop hairpin structure (Ashley et al. 2011; Fiedler et al. 2010; Wei et al. 2009).

Cellular Targeting and Delivery

A wide spectrum of ligands serving various purposes is conjugated to VLP surface which includes peptides, glycans, receptor specific ligands like folate and transferrin, antibody fragments and aptamers (Park et al. 2011). In the report of Ashley et al., MS2 VLPs have been modified to co-display the peptide (SP94) and a histidine-rich fusogenic, cell-penetrating peptide (H5WYG) (Ashley et al. 2011). SP4 imparts the particles 10^4 fold higher avidity for human hepatocellular carcinoma (HCC) and H5WYG promotes endosomal escape, thus make the particle suitable for target specifically delivery of encapsidated cargo molecules. H5WYG owing to the presence of protonatable secondary and/or tertiary amine groups can induce proton absorption across the endosomal membrane from cytosol resulting in swelling from an influx of water which ultimately leads to rupture of endosomal membrane and drug release. On the other hand, P22 and CPMV were functionalized with HIV-Tat (Anand et al. 2015) and arginine rich R5 peptides respectively (Anand et al. 2015; Wu et al. 2012). DNA aptamers against protein tyrosine kinase 7 receptors, expressed on Jurkat leukemia T cells have been reported to functionalize MS2 VLPs by Matt Francis group (Tong et al. 2009). MS2 VLPs functionalized with transferrin on its surface have been used to selectively deliver functional siRNA into HeLa cells by receptor mediated endocytosis (Galaway and Stockley 2013).

Stability

VLP structure is highly uniform. VLP coat proteins are arranged in a perfectly defined geometry which gives them a highly organized structure with very a lesser degree of variability (Rohovie et al. 2017). Stability of any kind of NPs is a major

issue of concern, mainly because of safe transport and adequate release of encapsulated drugs. Similar to other NPs a great length of research efforts is particularly dedicated to investigating different parameter controlling the stability of VLPs. With the development of various approaches of surface engineering, it was observed that multimodal functionalization has an impact on the stability of VLPs. For example, elucidation of the replication cycle of hepatitis B virus revealed that the stability of the viral assembly is conferred by the nucleic acid cargo, lipid and surface coating proteins. This may be reason why empty core protein particles show poor stability (Lu et al. 2015). On the other hand, genetic combination of antigen leads to misfolding of the antigen or the capsid proteins which in turn may either impair or completely abolish their function, disrupting the particle assembly (Rohovie et al. 2017). Incorporation of non-natural amino acids into VLP or antigen brings stable linkage but may accompany side reaction of azide/alkyne/tetrazine groups (Sasmal et al. 2012; van den Bosch et al. 2013; Versteegen et al. 2013) and misreading of non-natural amino acids (Aerni et al. 2015). Moreover, surface bound ligands often have been shown to alter the thermal stability also. In order to address the stability issues, various approaches have been adopted. Lu et al. introduced artificial covalent disulphide bridges to stabilize the HBVc VLP assembly (Lu et al. 2015). This group also replaced a surface spike with a natural mutant Q8N6M7 which helped in reducing systemic immunogenicity. Disulphide linkages also increase the dissociation temperature. Similar to other NPs, PEGylation of VLPs has also been studied as this method was shown to assist in avoiding immune clearance. PEGylated MS2 VLPs worked similarly but their retention in spleen was significantly reduced (Farkas et al. 2013). Further work showed that CD47 ectodomain or CD47 'self-peptide' also can be used to avoid immune system (Rodriguez et al. 2013).

Although VLP-based targeted therapeutic delivery is still in its infancy and a lot more developments are still required to make it a potential commodity with significant clinical value, promising research progress in this field has already been accomplished. Several advantages associated with this relatively young and versatile drug delivery platform have already stirred a genuine interest over improving its efficacy. Some of these advantages are as follows: (1) they can be produced in multiple copies by CFPS; (2) they are stabilized by disulphide bonds which will get reduced only inside the cytosol, resulting in disintegration of the assembly and release of the therapeutics; (3) they can be specifically targeted by functionalizing the surface with antibody fragments, etc.; and (4) while polymeric NPs usually show a slow and controlled drug release profile with prolonged period of time, the VLPs, once internalized into the target cells, undergo rapid eruption of its cargos (Rohovie et al. 2017; Lu et al. 2015; Yan et al. 2015). Each mode of drug release has its own benefits. Talking specifically about delivering therapeutic nucleic acids, use of VLPs in comparison to another nanoparticle system is rather limited. Among the different cargo molecules, VLPs have been best characterized for delivery of vaccines mainly. Despite this, few VLP-based applications of successful delivery of RNAi machinery and antisense oligonucleotides have been reported which we also have summarized here. All the functionalities, advantages, chemical modifications and cellular targeting strategies which have worked fruitfully for other systems

should also be translated to the delivery of nucleic acids for improving their therapeutic potential. Additionally, progress should be made with the focus of improving extravasation from blood vessels in order to increase tissue accumulation and reduce clearance as well as off target adverse effects.

Gold Nanoparticles (AuNPs)

AuNPs have been widely used to deliver gene because of their ease of formation, size diversity, surface functionalization and biocompatibility (Sperling et al. 2008). Besides these, AuNPs can be synthesized in a wide array of shapes and sizes by numerous approaches; the easiest and mostly followed process is reduction of gold salts, such as $HAuCl_4$ in presence of sodium citrate, formulated by Turkevich (Turkevich et al. 1951). AuNPs have long been used for RNAi delivery. The first application of AuNP to deliver siRNA was reported in 2006 by Oishi et al. They showed significant inhibition of luciferase expression in HuH-7 cells by transferring siRNA via AuNP (Oishi et al. 2006). It is interesting to note from this work that the intracellular delivery of AuNP-siRNA complex decorated with PEG does not require any transfection reagent. Entrapment of siRNAs with AuNPs is done mainly by two processes, namely, electrostatic interaction and layer by layer assembly (Hong and Nam 2014).

Similar to other nanoparticle system, AuNP also offers a wide variety of options of surface functionalization which increases their diversity of its application. Different chemical groups are anchored on the surface of AuNP via thiol (Au-S) linkers. Guo et al. developed charge-shifting AuNPs through layer-by-layer process where the surface of the nanoparticle was modified with negatively charged 11-mercaptoundecanoic acid (MUA) via Au-S bonding, followed by bPEI (25 kDa) deposition onto AuNP-MUA, forming cationic AuNP-MUA/bPEI core. Then the AuNP-MUA/bPEI core is sequentially layered with cis-aconitic anhydride-functionalized poly (allylamine) (PAH-Cit) and bPEI (25 kDa) where PAH-Cit polyanions contribute a charge-reversal reaction, helping the nanoparticle in turn, for endosomal escape (Guo et al. 2010). Very recently, diblock oligonucleotides with adenines have been employed as anchoring moieties for spatially controlled functionalization of AuNPs (Pei et al. 2012). Coating with polymers is another interesting option to augment transfection efficiency via AuNPs. Song et al. designed bPEI-coated gold nanoparticles for siRNA delivery. The AuNP-bPEI/siRNA complexes showed efficient knockdown of exogenous GFP and endogenous polo-like kinase 1 (PLk 1) in MDA-MB-435-GFP cells (Lee et al. 2011). We already have discussed how dendrimer-capped gold nanoparticles have been used for targeted gene delivery.

Quantum Dots (QDs)

Quantum dots are nanocrystals composed of semiconductor core, for example, CdSe, InP and PbSe enclosed in a shell of ZnS (Juzenas et al. 2008). Despite the lack of biocompatibility and risk of release of toxic metal ions due to surface oxidation, their use for gene delivery, specifically for siRNA-mediated gene silencing, is highly favoured because of extraordinary optical properties which enable the researcher to monitor the real-time effect of treatment (Sanvicens and Marco 2008). This problem of toxicity can be surpassed by coating the nanoparticle surface with biocompatible polymers. For example, β-CD-grafted L-arginine-conjugated quantum dot nanocarrier was developed as highly efficient siRNA delivery agent and imaging probe. The guanidium ion of L-arginine imparts positive charge on the surface enabling electrostatic binding of siRNA to it and β-CD molecules provide better protection to siRNA from external ribonucleases (Li et al. 2011). The same group designed a co-delivery strategy for both an siRNA and chemotherapeutic agent, doxorubicin (Dox), using quantum dot-β-CD-L-Arg (or, His) (Li et al. 2012). Similar to other nanoparticle system PEGylation to quantum dot surface also provides enhanced durability and metallotoxicity. In addition to this PEG layer also increases dispersibility and creates suitable docking sites for diverse group of biologically important molecules like fluorescent dyes, targeting ligands etc. (Hong and Nam 2014) A lot more research is required to curb down the negative effects of quantum dot in order to become more efficient gene delivery agent.

Carbon Nanotubes (CNTs)

Carbon nanotubes are one-dimensional hollow cylindrical structure with a typical diameter of 1–2 nm and length from 50 nm up to 1 cm. Because of their tubular morphology they are easily internalized by host cells. In addition to this, they permit encapsulation of molecules and provide material storage functions as well as protection and controlled release of loaded molecules (Ji et al. 2012; Pantarotto et al. 2004). That's why they have triggered enormous interests as shuttle nanovectors for delivering siRNA and drug molecules. Their extremely large surface area, with every atom exposed on its surface, allows for ultra-high functionalization and loading capacities. Ammonium-functionalization and surface modification with cationic polymers such as PEI and poly (diallyldimethylammonium) chloride (PDDA) are commonly applied for simple electrostatic conjugation of siRNA to their surface. They are mainly of two types, (i) single walled and (ii) multiwalled (Draz et al. 2014; Liu et al. 2007b). Zhang et al. proposed SWCNTs as an efficient vector for siRNA to suppress murine telomerase reverse transcriptase expression in murine tumor cells on both in vitro and in vivo levels (Zhang et al. 2006). MWCNTs are structurally less stable and chemically less reactive in comparison to SWCNTs. High transfection efficiency for MWCNTs can be achieved by various surface functionalization. For example, Liu M et al. developed polyamidoamine-functionalized

multiwalled carbon nanotubes (PAA-g-MWNTs) which showed effective gene delivery with low cytotoxicity (Liu et al. 2011b).

A relatively new carbon-based nanostructure graphene oxide generated huge interests as promising gene carrier. We already have discussed about dendron coated Graphene oxide. Graphene oxide shows π-π-stacking interactions which helps in enhanced drug loading and their controlled release (Yang et al. 2008). Zhi et al., taking the advantage of this property, developed a polyethylenimine (PEI)- and poly(sodium 4-styrenesulfonates)-conjugated graphene oxide nanocomplex for simultaneous co-delivery of microRNA-21 (mir21) and adriamycin (ADR), an anti-cancer drug which showed effective reduction of antitumor resistance in MCF-7/ADR, an adriamycin-resistant breast cancer cell line (Zhi et al. 2013).

Considerable technological success and theoretical understanding about the surface engineering of nanoparticle system have welcomed a new paradigm of gene delivery strategy through which the intrinsic limitations of conventional chemotherapy can be circumvented. Apart from cancer, several other genetic diseases are gradually being sought to be treated through nanoparticles. Several breakthrough researches have already revolutionized nanomedicine over past decades, theranostics being one of the youngest developments. Despite this extraordinary success in the past few years, the broader range of acceptance of nanoparticle-mediated gene therapy is limited mainly because of the toxicity issues and additional challenges associated along the delivery route like biodistribution, retention, etc. More researches are required to develop newer variety of nanocarrier systems, optimized to deliver siRNAs specifically to less accessible or hard-to-transfect tissues. Along with this, significant research efforts should be oriented on the betterment of existing coating polymers or developing novel polymers which are capable of to impart better pharmacokinetic properties to the nanoparticle systems, thus helping in providing better shielding, higher retention time and enhanced target specific tissue distribution etc.

11.7 Conclusion

The scope of improvement for each delivery system was discussed for each section. In order to achieve successful nucleic vaccine delivery to the target site, an efficient delivery system passing the extracellular and intracellular barriers needs to be developed. This can be possible by using a suitable physical, chemical or biological agent (Jorritsma et al. 2016). Each delivery system has its own advantages and disadvantages. Even though viral-based vectors are efficient, their low loading capacity and high production costs make them limited to in vitro studies. Polymeric and metal-based nanoparticles can be more suitable for nucleic acid delivery as they can be easily tailored with chemical modifications. The current chapter discusses more about the successful examples of in vitro work. The results from in vitro studies for most of nucleic acid delivery vectors were promising enough to encourage towards further step of conducting in vivo studies and clinical trials. Hence, research is

progressing towards in vivo application of nanoparticles. However, till now no nanoparticle-based nucleic acid delivery system was approved by FDA. Many more clinical trials are ongoing with a hope of success.

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Chapter 12
Plasmonic Hybrid Nanocomposites for Plasmon-Enhanced Fluorescence and Their Biomedical Applications

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Contents

Abstract Fluorescence is a powerful tool in biochemistry, biophysics, forensic science, and biotechnology. Two main principal properties for any fluorophore, brightness and photostability, are fundamentally important to achieve a high level of sensitivity for detection. Therefore, improvements in the technique are strongly

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encouraged and pursued, such as new developments in terms of the technique sensitivity, the range of fluorophores, their stability, and the versatility of the experimental setups that help move this particular scientific research in biosensing and molecular imaging forward. Therefore, a new avenue is based on the use of plasmonic nanostructures in the enhancement of the collective photo-physical properties including their absorption and fluorescence, known as "plasmon-enhanced fluorescence." Such plasmonic enhancement is due to the localized surface plasmon resonance at the metal surface, which leads to increasing the exciton radiative recombination rate in the fluorophore and thereby improves the signal obtained and increases sensitivity. In addition, the plasmonic enhancement might depend on several parameters such as nanoparticle size and shape, metal type, and the spectral overlap in the absorption spectra and the type and the separation distance between both plasmonic nanoparticle and the fluorophore. Throughout this chapter, previous approaches are discussed, which are devoted to tracking the influence of plasmonic nanostructures on the photoluminescence of the fluorophores especially the hybrid nanocomposites based on plasmonic/quantum dots including semiconductor and carbon-based nanoparticles. In addition, the possible applications of metal-enhanced fluorescence nanohybrids in the biological and medical applications such as imaging and biosensing techniques.

Keywords Metal-enhanced fluorescence · Plasmonic nanostructures · Fluorophores · Carbon dots · Quantum dots · Hybrid nanocomposites · Biosensing · Biomedical imaging

12.1 Introduction

Fluorescence spectroscopy is a widely employed technique for chemical analysis, biochemistry, biophysics, forensic science, biosensing, and biotechnology because of its inherent high sensitivity, and its large linear concentration ranges, often significantly larger than in absorption methods, but the latter find more applicability as relatively few species exhibit fluorescence (Skoog et al. 2017; Lakowicz 2013). Recently fluorescence has become a primary methodology in life sciences because of its sensitivity, ease of use, and versatility (Xie et al. 2008). It has been used as an imaging tool in the clinical diagnosis and monitoring processes in biological systems (Bardhan et al. 2009).

Particularly, molecular fluorescence is a luminescence process that occurs when an atom or molecule relaxes to its ground state, after being excited, by emitting light. A molecule that is capable of fluorescence is called a fluorophore. When light from an external source interacts with the fluorophore, the fluorophore absorbs the light energy, resulting in a higher energy state. As the excited fluorophore is unstable at higher energy states, it relaxes from its higher energy state to a meta-stable state via small non-radiative transitions and then finally releases its excess energy from the meta-stable excited state to the ground state via a radiative transition through the process of emission of light. The light energy emitted by a fluorophore

is always longer in wavelength than the light energy absorbed, due to some non-radiative energy loss during its transition to the ground state. Therefore, a lot of studies have been performed to achieve high fluorescence yields (Xie et al. 2008).

The understanding of the interaction of light with matter allows us to design and apply the mechanism into applications. Thus, it is necessary to properly understand how the light interacts with the matter and then design structures that allow optimum conversion of light into the specific application (Piccione et al. 2014). A wide range of methods has been developed for enhanced fluorescence to increase the sensitivity of fluorescence, such as optical fiber fluorescence detectors. Of all the methodologies, metal-enhanced fluorescence (MEF) has been the most widely investigated and explored. The attractive changes in fluorescent properties of fluorophores due to this MEF include increased rates of excitation, increased quantum yields, and decreased fluorescence lifetimes with an increased photostability. The presence of these metallic structures in the vicinity of the fluorophore can alter the optical properties of the fluorophore by increasing the excitation field depending on the distance between the metal nanoparticle and fluorophore (Geddes 2013).

Advancement in nanotechnology allows us to create nanoscale structures (Aslan et al. 2005; Rosi and Mirkin 2005; Katz and Willner 2004). As the size of the metal is reduced too much smaller than the wavelength of the incoming light, a localized collective oscillation of electrons occurs in metals (Lakowicz et al. 2004; Stoermer and Keating 2006). This is now commonly known as localized surface plasmon resonance (LSPR). This phenomenon has opened diverse opportunities in technology advancement, ranging from arts, science, medical, and engineering (Geddes et al. 2005; Touahir et al. 2010).

This chapter is devoted to exploring the photo-physical properties of plasmonic nanostructure based on the LSPR, in addition to the factors that determine the strength of LSPR such as the density of electrons, the effective electron mass, the shape, and size of the charge distribution. Furthermore, the influence of the LSPR on the fluorescence properties of the fluorophores such as organic dyes, quantum dots, and carbon dots has been demonstrated. In addition, the required criteria to achieve the metal-enhanced fluorescence phenomena has been discussed. Finally, an overview of the achieved work was done by our research group and others regarding using of engineered hybrid nanocomposites to achieve a MEF mechanism and their possible applications in the biomedical field such as biosensing and bioimaging.

12.2 Plasmonic Nanoparticles

12.2.1 Surface Plasmon

Surface plasmons originate from free collective charge oscillations on metallic surfaces. There are two types of plasmon modes on metallic surfaces, namely localized surface plasmons (LSPR) (Haes and Van Duyne 2002; Hutter and Fendler 2004; Willets and Van Duyne 2007) and propagating surface plasmons also referred to as

surface plasmon polaritons (SPP). LSPR are observed at an optical wavelength for subwavelength-sized particles, while surface plasmon polaritons are observed on flat or corrugated continuous surfaces (Pitarke et al. 2006).

Localized Surface Plasmon Resonance

LSPR is a subfield of plasmonics that is associated with resonances due to noble metal nanostructures which cause spectral absorption, scattering peaks, and strong electromagnetic (EM-field) near-field enhancements (Haes and Van Duyne 2002). Localized surface plasmon can be excited directly by an incident light beam. The oscillating electromagnetic field associated with the incident light interacts with the conduction electrons in the metal particle and displaces them with respect to the ionic lattice of the metal (**see** Fig. 12.1) (Mayer and Hafner 2011). Upon displacement of the electrons, an attracting force arises that pulls the electrons back into equilibrium. Thus the metal nanoparticle can be seen as an oscillating system, where the light represents an external force, which drives an oscillator. As a typical oscillating system, metallic nanoparticles exhibit resonance frequencies (Hutter and Fendler 2004; Willets and Van Duyne 2007). The resonance frequency (or resonance wavelength) is dependent on the size, the shape, the metal used, and the dielectric environment surrounding the metal nanoparticle. Highest fields at the surface of nanoparticles can be observed when the incident light has the same wavelength as the resonance wavelength (Haes and Van Duyne 2002).

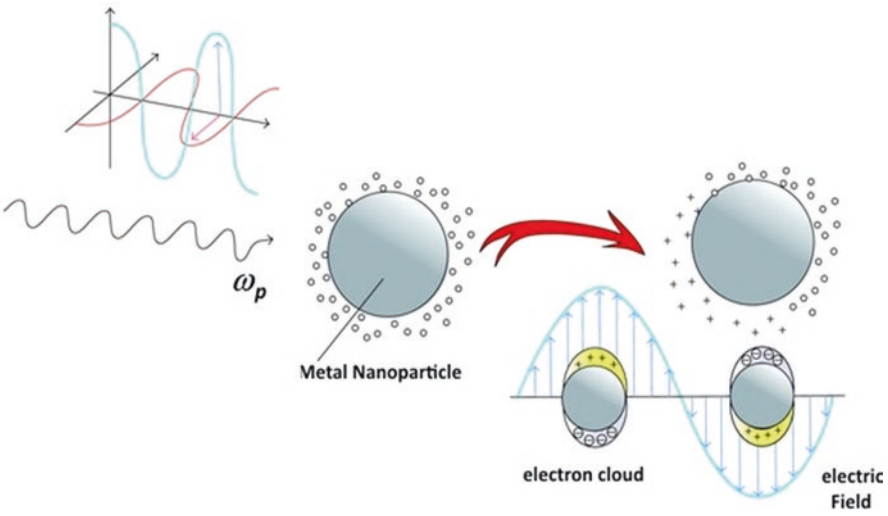

Fig. 12.1 Localized surface plasmon resonance (LSPR) of noble metal (Ag, Au) nanoparticles, a collective electron density oscillation caused by the electric field component of incoming light. (Reprinted with a Copyright permission from Anna Zielińska-Jurek 2014)

The interaction between those metal nanoparticles and the incoming light results can result in absorption of energy by the nanoparticles (generation of heat) or elastic scattering of light back to space (Mie 1908). Mie theory describes the EM-field enhancement within and out of the spherical particle and allows calculation of the scattering cross section $\sigma_{sca,}$ absorption cross section σ_{abs}, and extinction cross section σ_{ext}. The scattering cross section describes the ability to scatter the incident light into different directions with respect to the incident plane wave, while the absorption cross section describes the absorption of energy within the particle. The extinction cross section, also called the total cross section, is given as the sum of both:

$$\sigma_{ext} = \sigma_{abs} + \sigma_{sca} \tag{12.1}$$

In case of the spherical particles, both of $\sigma_{sca,}$ and σ_{abs} are given by:

$$\sigma_{abs} = kIm[\alpha] = 4\pi k a^3 \times Im[g_d] \tag{12.2}$$

$$\sigma_{sca} = \left(k^4 / 6\pi\right) I\alpha I^2 = \left(8\pi / 3\right) k^4 a^6 I\, g_d I^2 \tag{12.3}$$

Where $\boldsymbol{k} = 2\pi / \lambda$, $\boldsymbol{\alpha}$ is the dipole polarizability that equal to $4\pi g_d a^3$, and $\boldsymbol{g_d}$ is the asymmetrical term for a dipole that equals to $\varepsilon_i - \varepsilon_m/\varepsilon_i + \chi\varepsilon_m$. In addition, χ is a shape-dependent parameter which equals to 2 for a sphere and can be larger (smaller) for other shape. This means that the extinction cross section (σ_{ext}) as a function of LSPR frequency could be given by:

$$\sigma_{ext}(\omega) = 9\frac{\omega}{c}\varepsilon_m^{3/2} V_0 \frac{\varepsilon_{i,2}(\omega)}{\left|\varepsilon_{i,1}(\omega) + 2\varepsilon_m\right|^2 + \varepsilon_{i,2}(\omega)^2} \tag{12.4}$$

Where V_0 is the volume of spherical shape that equal to $4\pi R^3/3$. The optical cross section against the actual physical geometrical cross section of the sphere, a dimensionless optical efficiency is used:

$$Q_{ext} = \frac{\sigma_{ext}}{\pi a^2}; Q_{abs} = \frac{\sigma_{abs}}{\pi a^2}; Q_{sca} = \frac{\sigma_{sca}}{\pi a^2} \tag{12.5}$$

This presents a problem in identifying small particles from a background with larger particle sizes. By changing the property of the LSPR of the metal nanoparticle, the optical efficiencies can be tuned and it is possible to achieve absorption efficiency larger than 1 (Nagel and Scarpulla 2010), i.e., more energy is being absorbed per unit area. This is beneficial for applications such as where the heat energy absorbed is used to convert to another useful energy form such as electricity for solar cell or water splitting.

Factors that Affect the Localized Surface Plasmon Resonance

The oscillation frequency of the surface plasmon band (SP) is determined by four factors: the density of electrons, the effective electron mass, the shape, and size of the charge distribution. The frequency and width of SPR depend on the size and shape of the metal nanoparticles as well as on the dielectric constant of the metal itself and the surrounding medium (Kreibig and Vollmer 1995; Link and El-Sayed 2003). The extreme sensitivity of LSPR to the particle size and shape makes it an attractive research subject because the resonance wavelength can be tuned to fit a specific wavelength of interest. The absorption and scattering cross section of the spherical particles is dependent on their size with scattering which becomes dominant as the size increases as shown in Fig. 12.2. For gold nanoparticles, it is found that diameter *D* is lower than 20 nm, and the absorption channel is dominant. As the size increases, the scattering becomes dominant (Tesler et al. 2011). Simulation based on the Mie theory and experiment results showed that increasing the size also causes red-shifting and broadening the LSPR spectrum due to phase retardation effects and presence of higher order mode. The strength and the distance of the induced electric field also increased when the particle gets larger (Hutter and Fendler 2004; Willets and Van Duyne 2007; Yeshchenko et al. 2012).

For an asymmetrical shaped particle, the effects of L-SPR become more complex. Some additional parameters to consider are the axis at which the size increases, and the direction and polarization of the incident light. For example, the SPR absorption in spherical Au and Ag NPs occurs at about 520 and 410 nm, respec-

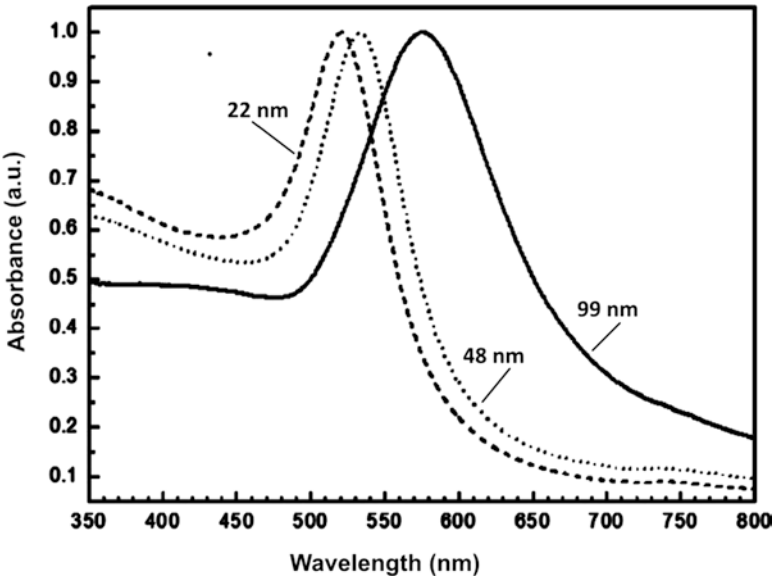

Fig. 12.2 Localized surface plasmon resonance (LSPR) of gold nanoparticles (Au NPs) dependent on the particle size. (Reprinted with a Copyright permission from Emam et al. 2017a)

tively (Fig. 12.3a). This absorption is absent for clusters (i.e., << 2 nm), as well as bulk Au. In the case of rod-shaped Au NPs, two absorption bands have been obtained (Emam et al. 2015). The first one which appears at ~ 520 nm corresponds to the oscillation of the electrons perpendicular to the long rod axis and is called transverse localized surface plasmon absorption (T-LSPR), which is insensitive to the nanorod length but coincides with the LSPR band of the spherical-like shapes (Emam et al. 2015; Henson et al. 2009). In addition, the second absorption band known as the longitudinal LSPR band that appears at a lower energy is caused by the oscillation of the free electrons along the long rod axis (Fig. 12.3b). Such band, the LSPR, is very sensitive to the aspect ratio (length/width) of the rods where a redshift occurs as the aspect ratio increases (Emam et al. 2015).

Other than the major change in the extinction spectrum across different shapes, a tremendous increase of electric field enhancement is found at the sharp edges. With this objective in mind, highly complex asymmetrical structures such as nanorice and nanostars deposition (Brand et al. 2006; Liu et al. 2013; Wu et al. 2009; Homan et al. 2011) are usually polycrystalline (Rodríguez-Oliveros and Sánchez-Gil 2012; Kumar et al. 2007) and become a subject of huge interest. For an example the LSPR band of triangular-shaped plasmonic nanoparticles split into three bands, longitudinal mode, transverse bands (i.e., in-plane dipole resonance

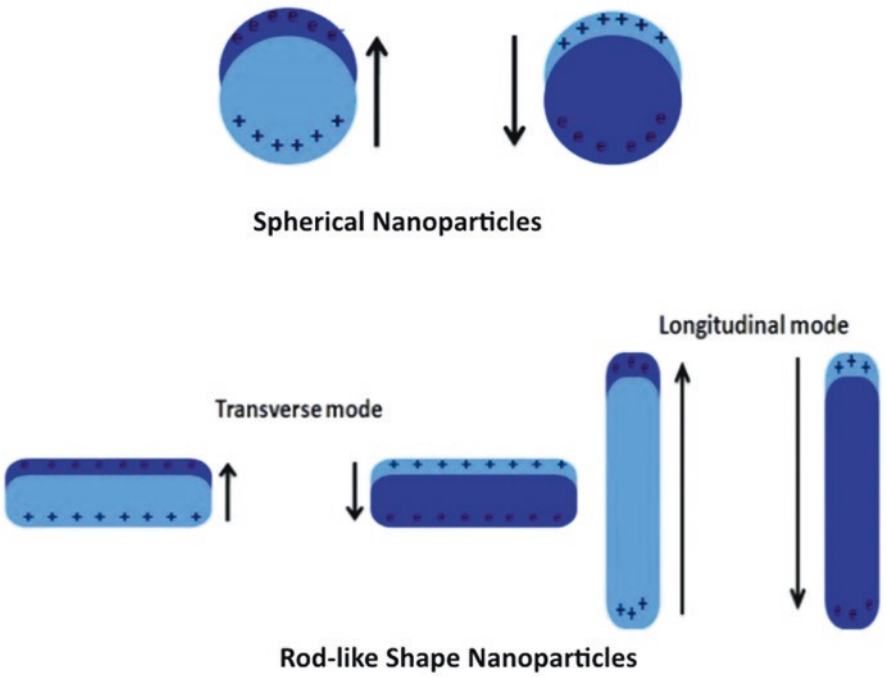

Fig. 12.3 Schematic representation of SPR excitation for spherical and rod-like shapes of gold nanoparticles. (Reprinted with a Copyright permission from Jayabal et al. 2015)

mode), and quadrupole bands (i.e., in-plane quadrupole and out-of-plane quadrupole resonances modes), as shown in Fig. 12.4 (Jin et al. 2001, 2003; Millstone et al. 2005; Callegari et al. 2003; Sherry et al. 2006). In such case, the maximum enhancement for the dipole resonance is at the tips. While for the quadrupole resonance, the regions for localized field enhancement are allocated at the sides. Afterward, the quadrupole band decayed away from the surface much faster than the dipole band around the particles tips, as shown in Fig. 12.4 (Kelly et al. 2003).

It is well-known that the dielectric constant of the surrounding media such as solvent or capping materials affects the SPR of metallic nanoparticles. Such an effect was attributed to the alteration in the ability of the surface to accommodate the electron density of the nanoparticles (Eustis and El-Sayed 2006; Jain et al. 2007). However, the capping material is the most important in determining the shift of the plasmon resonance. It is possible to shift away from the resonance peak from the interband transition by choosing an appropriate embedding medium. The dielectric constant of the surrounding medium determines the value ϵ and therefore the wavelength at which the resonance occurs. Medium with the higher dielectric function will cause further redshift to the resonance peak. Consequently, any chemically

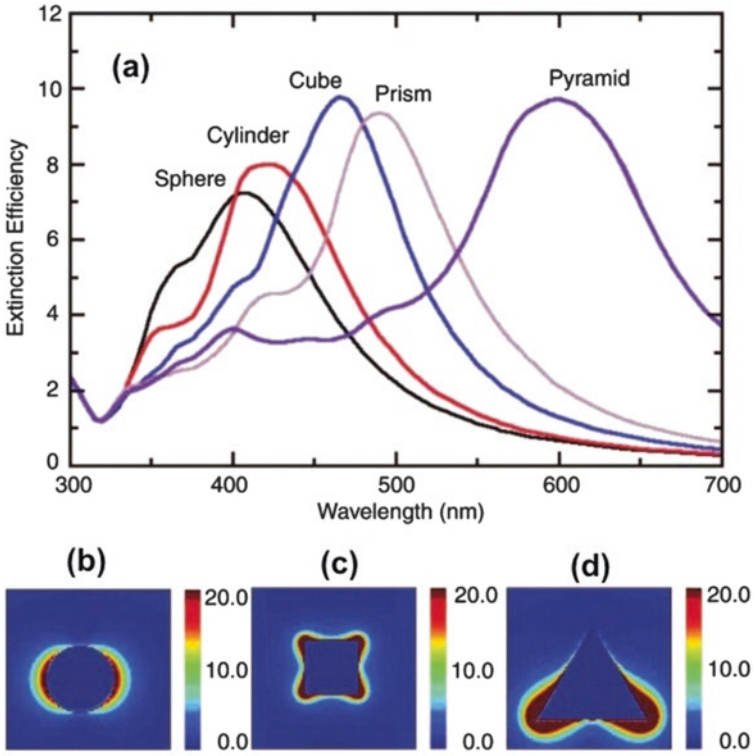

Fig. 12.4 (**a**) Extinction efficiency of silver nanoparticles in vacuum with same volume as of a 50-nm radius sized sphere but different shapes. The simulated electoral field contour map for corresponding (**b**) sphere, (**c**) cube, and (**d**) pyramid. (Reprinted with a copyright permission from Haes et al. 2005)

bonded molecules can detect the change in the electron density on the surface, which results in a change in the position of surface plasmon absorption band (Eustis and El-Sayed 2006). In metal nanoshell, the core material could be dielectric or semiconducting, whereas the shell material could be metallic nanoparticles. In these hybrid nanostructures, the SPR is strongly dependent on the relative thickness of the nanoparticle core and its metallic shell. Therefore, the position of the plasmon band can be tuned anywhere across the visible or infrared regions of the optical spectrum, by varying the core and shell thicknesses as shown in Fig. 12.5 (Jain et al. 2007; Oldenburg et al. 1998; Yu et al. 2017; Prodan et al. 2003; Jain et al. 2008; Ghosh Chaudhuri and Paria 2011).

Whereas, in the case of alloyed nanostructures, the position of the SPR absorption band is linearly dependent on their chemical composition (Link et al. 1999). Therefore, a strong redshift SPR band could be observed upon mixing of plasmonic nanostructures with other materials (e.g., magnetic or semiconducting materials) within the same nanoobject (Ghosh Chaudhuri and Paria 2011; Shi et al. 2006; Lee and El-Sayed 2006; Barcaro et al. 2015; Ferrando et al. 2008). As reported by Girgis et al. and Emam et al., this redshift in Au-Co compared to pure gold nanoparticles is due to the homogeneous mixture of the metal-metal bond between the alloys and constitutes such as gold and cobalt leading to the formation of an intermetallic or alloyed structure. In this case, Co^{2+} ions were diffused into the gold nanoparticles host crystal (Girgis et al. 2012), as shown in Fig. 12.6. Consequently, the alteration in the SPR for the host crystal gold nanoparticles via electronic charging or loss of

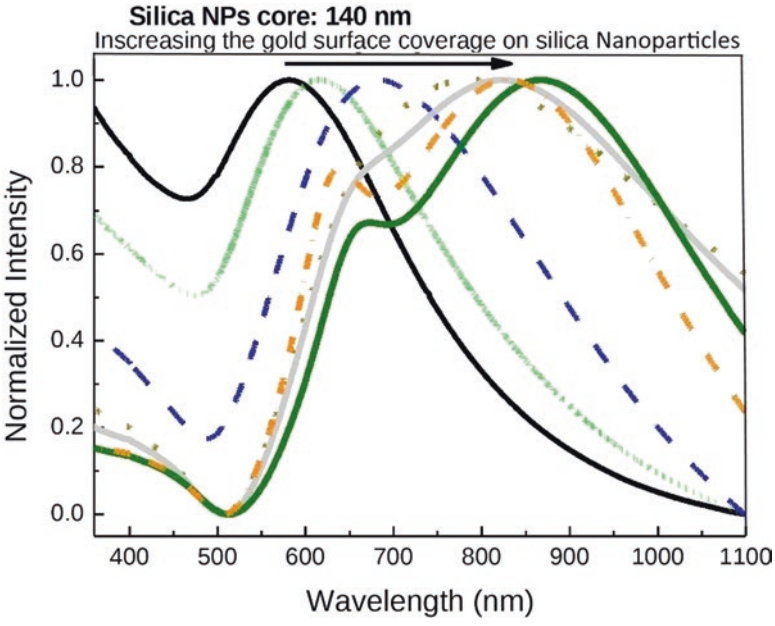

Fig. 12.5 Redshift in the absorption spectra of silica-gold core-shell nanoparticles with increasing in the gold nanoshells on silica nanoparticles. (Reprinted with permission from Lien et al. 2014)

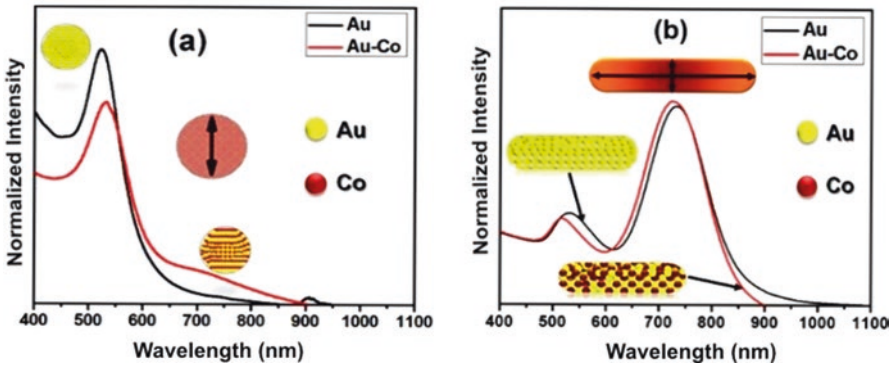

Fig. 12.6 (**a**) Redshift surface plasmon absorption band in case of spherical gold-cobalt nanoalloys compared to pure gold nanoparticles (**b**) Blue shift in the surface plasmon absorption band in case of rod-like shape of gold-cobalt alloyed nanoparticles. (Reproduced with permission from Emam et al. 2015)

continuous density of states causing a plasmon band shift (Emam et al. 2015; Xu et al. 2007; Boyer et al. 2010).

12.3 Metal-Enhanced Fluorescence

The fluorescence emission of fluorophores can be enhanced by attaching them to materials that exhibit plasmon resonance, commonly known as metal-enhanced fluorescence (MEF) (Geddes 2010, 2013; Geddes and Lakowicz 2002; Xie et al. 2006; Deng et al. 2013). This is also sometimes referred to as plasmon-enhanced fluorescence (PEF) (Bauch et al. 2014; Gandra et al. 2014; Feng et al. 2015). In MEF, the emission is enhanced owing to a strong localized field enhancement that is near the metal surface because the surface plasmons are being excited by the light. Through the interaction of the fluorophore molecule with the metal surface, decay rates for the fluorophore are altered which leads to fluorescence enhancement (Morton et al. 2011). The strength of the MEF depends mainly on spectra overlapping of the excitation and emission of the fluorophores to the plasmon resonance wavelength of the metal (Geddes 2010; Chen et al. 2007; Bharadwaj and Novotny 2007; Emam et al. 2017b), location of hot spots (Yuan et al. 2013), and the metal-fluorophore distance (Gandra et al. 2014; Emam et al. 2017b; Zhou et al. 2014; Mishra et al. 2013).

There are two main processes that give rise to MEF: First of them is the external E-field that influences the molecules and the second one is based on the emission of radiation influenced by local field environment. When a fluorophore is in the vicinity of a plasmon resonating nanoparticle, the fluorophore will experience the E-field generated by the nanoparticle. Those enhanced electric fields increase the amount of energy absorbed by the fluorophore known as excitation enhancement. The rate of

the enhanced excitation field (E_{ex}) can be expressed into the following relationship:

$$E_{Ex} = \frac{\left|\boldsymbol{E}\left(x_d, \lambda_{ex}\right) \cdot \boldsymbol{p}\right|^2}{\left|E_i\right|^2} \tag{12.6}$$

Where (x_d, λ_{ex}) is the electric field at the position and wavelength of excitation, ŭ5D; is the emitters (fluorophore in this case) orientation, and E_i is the incident free space electric field without the presence of nanospheres. In MEF, the electromagnetic coupling between the fluorophore and the nanoparticle plasmon also causes an increase in the radiative decay rate of the molecule at the emission wavelength or decreases the decay rate if quenching occurs. This emission enhancement introduces new radiative and non-radiative decay rates (Γ_m *and* $\Gamma_{m,nr}$), and modifies both the quantum yield and lifetime of the fluorophore as follow:

$$Q_0 = \frac{\Gamma_0}{\left(\Gamma_0 + k_{nr}\right)} \leftrightarrow Q_m = \frac{\left(\Gamma_{nr} + \Gamma_{0,r}\right)}{\left(\Gamma_{m,r} + \Gamma_{0,r} + \Gamma_{m,nr} + k_{nr}\right)} \tag{12.7}$$

$$\tau_0 = \frac{1}{\left(\Gamma_r + k_{nr}\right)} = \frac{Q_0}{\Gamma} \leftrightarrow \tau_m = \frac{1}{\left(\Gamma_{m,r} + \Gamma_{0,r} + \Gamma_{m,nr} + k_{nr}\right)} \tag{12.8}$$

Where Q_m is the modified quantum yield due to MEF, the subscript ŭ5A; represents a modified term due to the plasmon coupling. The ability to modify the quantum yield of the fluorophores is an important benefit for MEF because fluorophores with poor quantum yields can be improved externally through coupling with SPR generated by the metal (see Fig. 12.7).

In such case, the final emission will be enhanced, which is given by:

$$E_m = \frac{Q_m}{Q_0} \tag{12.9}$$

Together, the new excitation and decay rate increased the rate of total fluorescence emission (E_F), and is related by the following relationship:

$$E_F = E_{Ex} E_{Em} \tag{12.10}$$

It is worth emphasizing that from the above equations, the MEF is primarily due to (1) E-field enhancement which boosts the excitation rate of the fluorophores, and (2) the addition of new radiative decay channels that improve the emission rates and quantum yields. In the case where the fluorophore has an intrinsic high quantum yield, the emission enhancement will not be significant. Several parameters and criteria must be taken into consideration to be useful in the fabrication and engineering of metal-enhanced fluorescence-based hybrid nanocomposites. These parameters include *(i)* the degree of spectral overlaps between the emission spectra of the fluorophore and

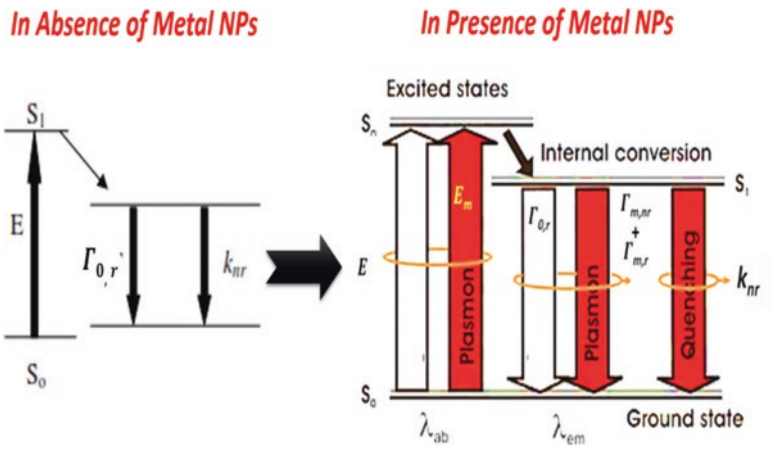

Fig. 12.7 A simplified Jablonski diagram showing the additional decay routes in both of presence and absence of plasmonics nanostructures. (Adopted from Bauch et al. 2014)

LSPR spectra (Geddes 2010; Chen et al. 2007; Lakowicz 2005) and *(ii)* the fluorophores should all be located at region of "hot spots" where electric field generated by SPR is the highest (Aslan et al. 2005; Yuan et al. 2013; Hrelescu et al. 2011; Fales et al. 2011). Finally, metal-fluorophore distance is widely recognized that the MEF is highly dependent on the distance between the fluorophores and the metal nanoparticles. Quenching occurs when the fluorophores are too close to the metal and facilitate non-radiative energy transfer and dissipation of energy in the fluorophore-metal system (Gandra et al. 2014; Zhou et al. 2014; Mishra et al. 2013; Ray et al. 2006a; Dragan et al. 2012), (See Fig. 12.8). At distance below the optimum enhancement, the enhancement factor follows a ŭ5F;$^{-6}$. Whereas when the distance gets further away, the dipole near-field of the SPR drops ∝ 1/ŭ5F;^{3} (or $1/r^5$ for quadrupole) and thus weakened the enhancement (Zhou et al. 2014; Chatterjee et al. 2011). However, an optimum distance appears to depend on the surrounding medium and the plasmonic structures (Eustis and El-Sayed 2006; Zhou et al. 2014; Chatterjee et al. 2011; Dulkeith et al. 2005). For example, Li et al. demonstrated that the magnitude of the fluorescence enhancement in C-dots/Ag@SiO_2 hybrid nanocomposites increases as a function of metal-fluorophore distance by the adjusting of the silica spacer thickness (Li et al. 2012).

12.4 Engineered Hybrid Nanocomposites for MEF Effect

Since the discovery of metal-enhanced fluorescence (MEF), it has been receiving huge attention and soon becomes a very active research field. Leading by recent technology push from advancement in nanofabrication and characterization techniques, various fabrication techniques, materials, and structures have been explored progressively to obtain better MEF structures. Gold and silver are the primary candidates of interest due to their SPR in visible and NIR regions. Regardless, both metals can offer large

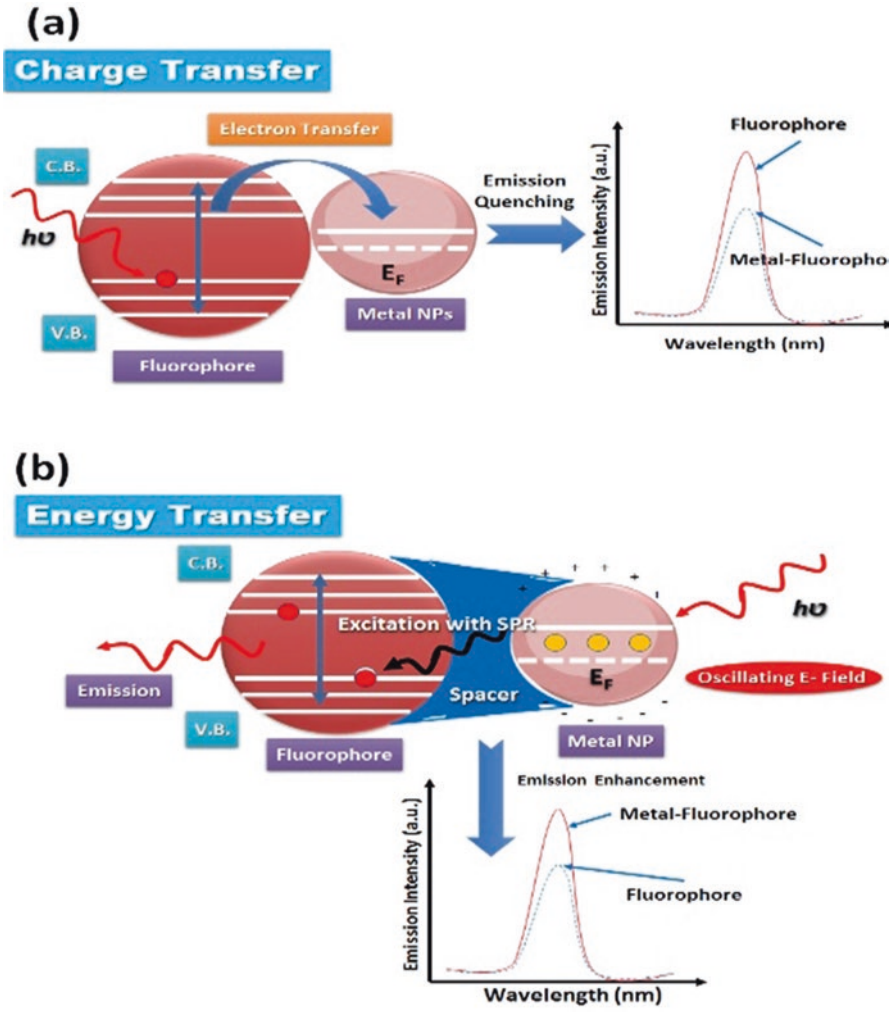

Fig. 12.8 A simplified schematic diagram shows the possible plasmonic enhanced fluorescence mechanisms (**a**) Quenching and (**b**) Enhancement

enhancement factors, and the signal is usually homogeneous throughout the substrate, which is important for biosensing and bioimaging applications (Deng et al. 2013).

In this section, an overview of the achieved work was done by our research group and others regarding using of engineered hybrid nanocomposites to achieve MEF mechanism. First of these studies is that achieved by Ragab et al. (Gadallah et al. 2013). They investigated the plasmonics effects of Ag NPs on the collective optical properties of fluorescein dye at different v/v ratios. In such study, a remarkable enhancement in the absorption and emission of fluorescein dye with an enhancement factor about threefold has been detected, as shown in Fig. 12.9. In addition, a significant increase in the rate of radiative decays was detected (Gadallah et al. 2013). These obtained enhancement mechanisms are attributed to a modification of

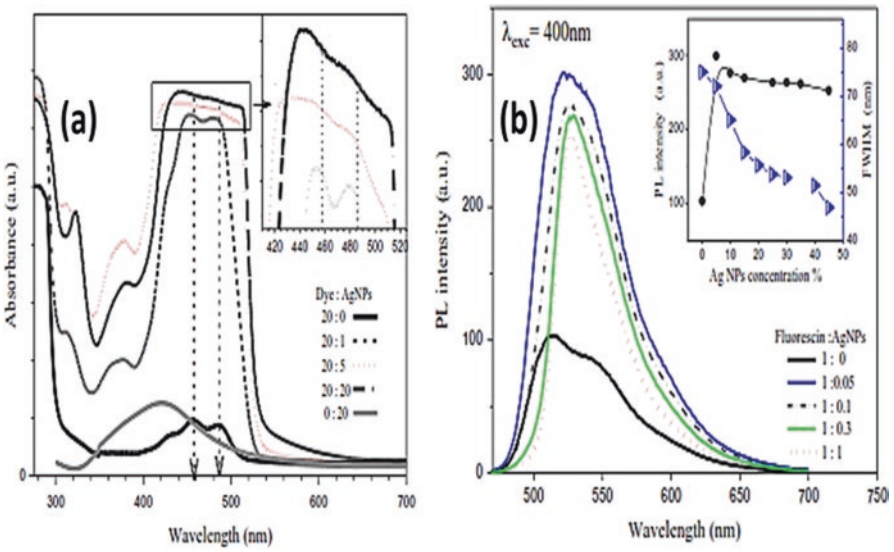

Fig. 12.9 (**a**) Absorption and (**b**) emission spectra of fluorescein dye, silver nanoparticles, and fluorescein: silver nanoparticles mixtures. (Reprinted with a copyright permission from Gadallah et al. 2013)

the local density of EM-modes in the vicinity of Ag nanoparticles at energies resonant with surface Plasmon (Xu et al. 2004).

In other studies, the influence of plasmonic nanostructures such as Au and Ag NPs has been investigated on the photo-physical properties of the semiconductor quantum dots such as CdSe and CdTe nanocrystals (Ragab et al. 2014a, b; Giba et al. 2015; Rady 2018). Ragab and co-workers demonstrated the influence of plasmonic silver nanostructures on the photo-physical properties especially the emissive (i.e., steady-state and upconversion) and laser spectroscopic properties of CdTe NCs (Ragab et al. 2014a, b; Giba et al. 2015). They reported in their studies a remarkable enhancement in the emission efficiencies upon the addition of Ag NPs at different CdTe:Ag NPs v/v ratios up to 11-fold, followed by a reduction in the radiative lifetimes (see Fig. 12.10) (Ragab et al. 2014a). This enhancement effect was attributed to energy transfer between the resonant (coupling) plasmonic field of Ag NPs and CdTe excitonic energy state. Although, no significant change in the upconversion spectrum either in the presence or absence of Ag NPs; an increase in both the absorption and emission rate of CdTe QDs was noticed.

Furthermore, Mansour *et al.* developed chemically a type of Plasmonic/Semiconductor such as Au/CdSe heterostructures of controlled morphology and their hybrid nanocomposites with graphene (Rady 2018; Mansour et al. 2017). Based on the photo-physical measurements, the presence of plasmonic nanocrystals such as Au NPs in direct contact with the semiconductor quantum dots such as CdSe QDs could enhance the optical absorptivity but quench their photoluminescence properties due to the charge transfer from the conduction band of the semiconductor to the Fermi level of the metallic part as shown in Fig. 12.11 (Mansour et al. 2017; Hsieh et al. 2007; Pons et al. 2007).

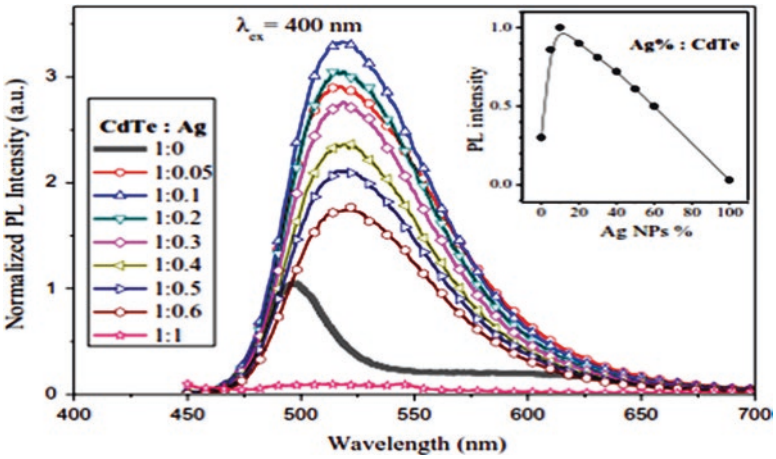

Fig. 12.10 PL of CdTe:Ag nanohybrids at different concentrations of Ag nanoparticles. (Reprinted with a copyright permission from Ragab et al. 2014a)

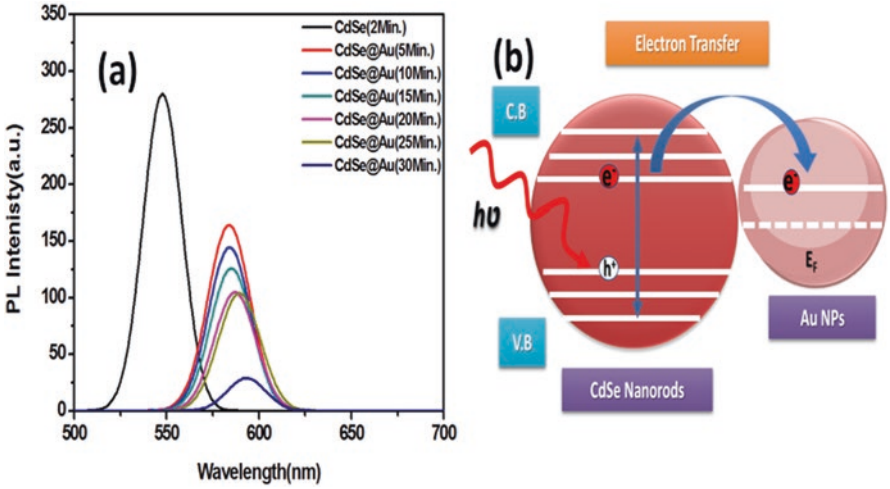

Fig. 12.11 (**a**) PLE spectra of Au/CdSe tetrapod-like shape heterostructure, (**b**) fluorescence quenching mechanism in Au/CdSe. (Reused from Rady 2018)

In contrast, besides the increase in the optical absorptivity, a remarkable enhancement of the quantum efficiency has been observed for the Au/CdSe heterostructures in presence of graphene (about ~ 4.5 to 12 fold intensity in the emission intensity) as shown in Fig. 12.12. This might be because the rate of the electron transfer from graphene to the metal is faster than that from semiconductor to the metal achieving the MEF effect in hybrid nanostructures based on metal/semiconductor heterostructures such as Au/CdSe tetrapods (see Fig. 12.13) (Rady 2018).

Finally, Emam et al. developed a novel fluorescent hybrid nanocomposite as an alternative to plasmonic/cadmium-based quantum dots such as plasmonic/C-dots. These hybrid nanocomposites include C-dots/Au and C-dots/Ag nanohybrids that

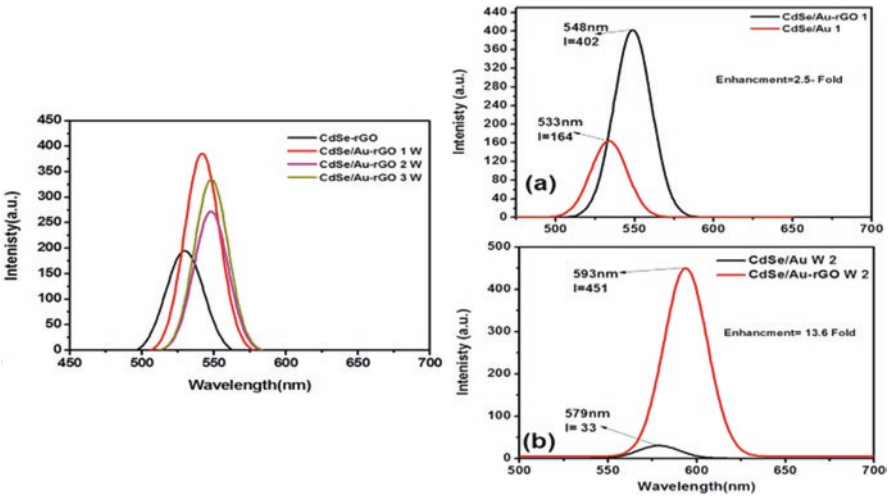

Fig. 12.12 PLE spectra of Au/CdSe tetrapod-like shape heterostructure in presence of rGO. (Reused from Rady 2018)

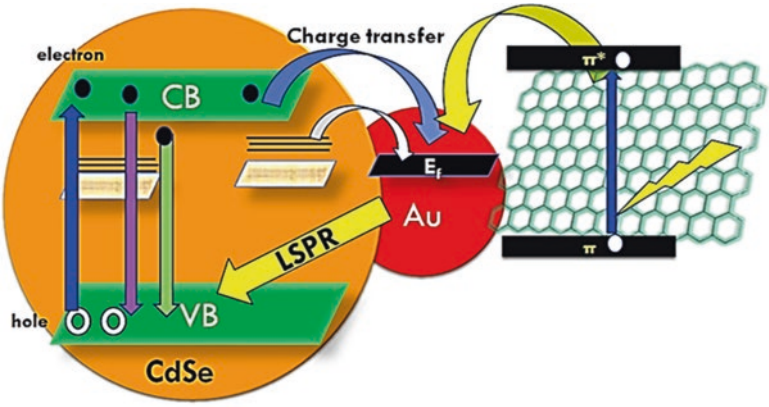

Fig. 12.13 Schematic diagram for PLE enhancement mechanism in Au/CdSe tetrapod heterostructure upon their loading on rGO. (Reused from Rady 2018)

are chemically prepared via microwave irradiation (MWI) method (Emam et al. 2017b; 2018). Remarkable enhancements in the collective optical properties and parameters such as absorptivity and fluorescence quantum yield (FL-QY), accompanied with the reduction in the rate of electron-hole recombination were observed for the hybrid nanostructure compared to pure C-dots (Emam et al. 2017b; 2018) as shown in Figs. 12.14 and 12.15 and Table 12.1.

This enhancement is due to enhancing the incident excitation field via L-SPR in metallic part, which leads to increasing the exciton radiative recombination rate in the carbon dots, which is dependent on the spectral overlap in the absorption spectra.

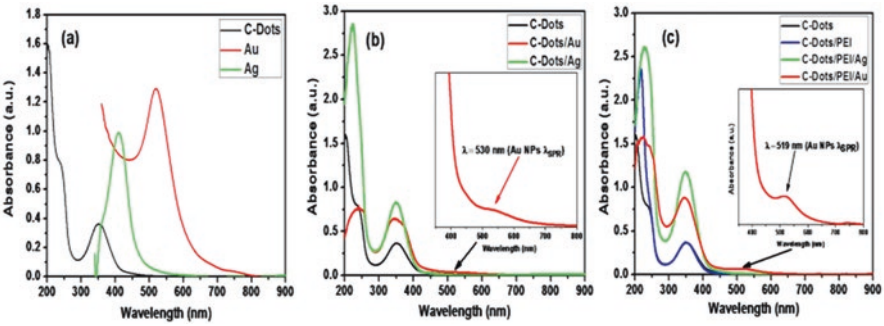

Fig. 12.14 Absorption spectra for each of (**a**) naked C-dots, Ag, and Au NPs, respectively. (**b**) C-dots, C-dots/Ag, and C-dots/Au nanohybrid, and (**c**) naked C-dots, C-dots/PEI, C-dots/PEI/Ag, and C-dots/PEI/Au nanohybrids. (Reprinted with a copyright permission from Emam et al. 2017b)

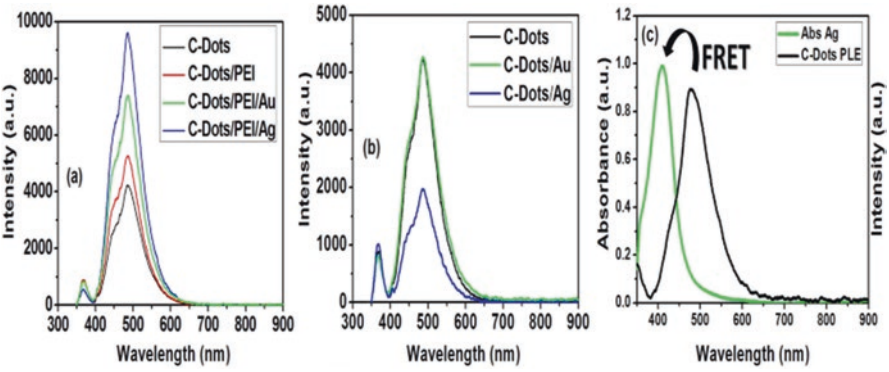

Fig. 12.15 Effect of plasmonic NPs on the PLE features of C-dots; (**a**) in the presence or (**b**) in the absence of spacer (i.e., PEI) upon excitation at steady-state condition (i.e., 366 nm). (**c**) Spectral overlapping between PLE spectra C-dots (black line) and the adsorption spectrum of Ag NPs (green line). (Reprinted with a copyright permission from Emam et al. 2017b)

Table 12.1 Influence of plasmonic nanoparticles (Au and Ag NPs) on the PLE properties of C-dots in presence or absence of polymeric spacer (i.e., PEI) (Emam et al. 2017b)

	At λ_{ex} [(a)] 366 nm			
Sample	I[(b)]	I/I_0[(c)]	QY/QY_{Dye} [(d)] (steady-state)	FWHM [(e)] (steady-state)
C-dots	(I_0) 4223.69	–	22.468	33.60484
C-dots/Au	4683.54	1.11	36.40	86.74477
C-dots/PEI/Au	7403.64	1.75	55.52	30.56937
C-dots/Ag	1833.45	0.434	8.723	2.5756916
C-dots/PEI/Ag	9134.23	2.16	77.213	17.44447

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[(a)]**λ_{ex}:** excitation wavelength. [(b)]**λ_{em}:** PLE wavelength. [(c)]**I/I_0:** relative enhancement factor. [(d)]**QY/QY_{Dye}:** relative quantum yield. [(e)]**FWHM:** full width at half-maximum

This plasmonic enhancement was more pronounced in the case of C-dots/Ag than that of C-dots/Au nanohybrids, due to low intrinsic loss and the degree of the overlap between the absorption spectra of AgNPs and C-dots. Furthermore, picosecond decay measurements show a decreased lifetime of C-dots in the presence of the plasmonic effect, due to the increased rates of radiative decay (see Fig. 12.16).

As shown in Fig. 12.17, the possible interactions between plasmonic material and fluorophores could be summarized as follows: (i) the excitation field can be enhanced through a coupling between the surface plasmon (SP)-assisted generated local field into the incident field. In such a case, plasmonic nanostructure could act as an optical concentrator for the incident source, resulting in a remarkable enhancement of optical absorption; furthermore, (ii) plasmonic nanostructures could be used as an excitation source to excite the fluorophore, as long as their SP energy is much higher than the band-gap emission of fluorophores (Achermann 2010; Sun et al. 2009; Hwang et al. 2009). Finally, iii) conversely to the previous pathway, PLE could be enhanced via an efficient energy transfer between the fluorophores and the plasmonic nanostructures when the exciton energy is greater than SP energy, which attributed to exciton-SP quadrupole interaction (Achermann 2010; Zhou et al. 2011; Cheng et al. 2010). Depending on the band structures of fluorophores (i.e. HOMO & LUMO) and plasmonic particles (i.e. Fermi level & Wave function), the PLE quenching or enhancement could be achieved (Deng et al. 2013; Achermann 2010; Sun et al. 2009; Hwang et al. 2009; Shevchenko et al. 2008; AbouZeid et al. 2011; Liaw et al. 2014; Zhang et al. 2007). If the C-dots based on fluorophores are located in a close proximity to the metallic surface, a non-radiative dumping is due to either energy transfer between the C-dots and the metal or the electron transfer from C-dots to the metal (Fig. 12.17a) (Emam et al. 2017b, 2018). Whereas the C-dots/

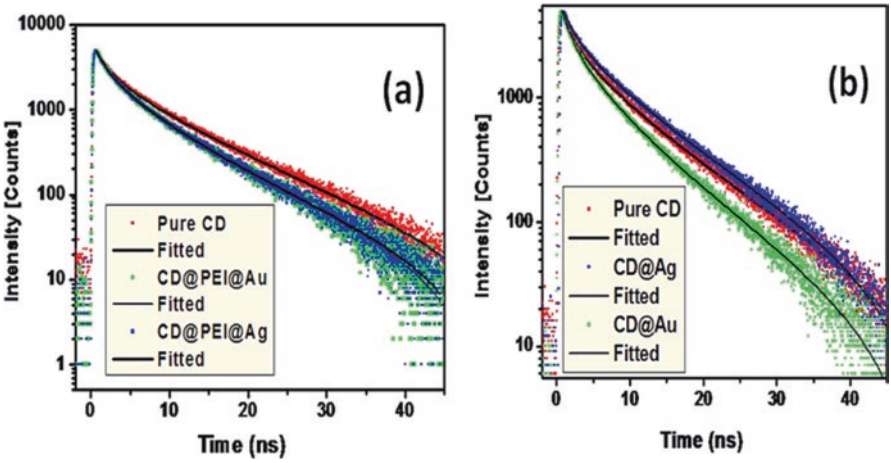

Fig. 12.16 Effect of plasmonic nanostructures (i.e., Ag and Au) on the radiative decay of the C-dots in presence (**a**) and absence (**b**) of dielectric polymeric spacer. (Reprinted with a copyright permission from Emam et al. 2018)

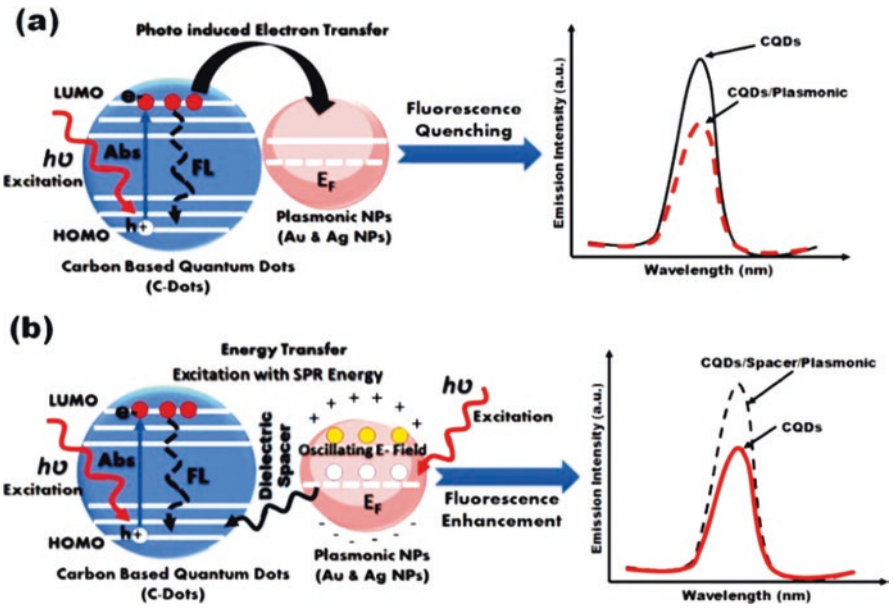

Fig. 12.17 Schematic diagram for (**a**) enhancement and (**b**) quenching mechanism C-dots/plasmonic. (Reprinted with a copyright permission from Emam et al. 2017b)

plasmonic hybrid nanostructures are separated with a dielectric spacer such as polymer (i.e., distance increase), light–mater interactions will be enhanced near the metal surface based on the enhancement of local fields associated with the SP of the metallic part. This effect could enhance the fluorescence of the C-dots based on fluorophore part as significantly shown in Fig. 12.17b (Hsieh et al. 2007; Pons et al. 2007; Ran et al. 2014).

12.5 Biomedical Applications of Engineered Metal-Enhanced Fluorescence Nanosystems

12.5.1 Biosensing

During the last decade, metal-enhanced fluorescence (MEF)-based engineered hybrid nanocomposites have been used in the fabrication of biosensor nanosystems to improve the sensitivity of fluorescence detection to detect molecules/moieties (Lee et al. 2011; Xu et al. 2017; Jeong et al. 2018) and heavy metals (Peng et al. 2018) at ultra-low concentrations. Along with signal enhancement, this promising technology allows advanced biological analysis for specified biomarkers and bioimaging on an adequate design.

As previously mentioned above, the MEF process depends on several critical parameters to induce desirable effects, consequently introducing a new trend in fluorescence detection. Therefore, it is essential to use brighter and more photostable fluorophores to achieve a high level of sensitivity of the biosensor. In MEF-based biosensors, the presence of metal near the fluorophore increases the rate of excitation and emission by opening additional electron configurations of fluorophores. In addition, it increases the photostability and emissive properties (i.e. fluorescence quantum yield, FLQY) of fluorophores compared to other conventional fluorophore-based biosensor (Feng et al. 2015; Emam et al. 2017b; Li et al. 2012; Emam et al. 2018; Khurgin et al. 2007; Lakowicz et al. 2008; Ray et al. 2006b). Furthermore, MEF-nanosystems provide an advantageous method for fabrication of biosensing platform. Such platform that can combine between the sensing transducers fluorophores and a plasmonic-based amplifier for the resultant signal within a single system, compared to traditional biosensors (see Fig. 12.18). Thus, these features explain why MEF is beneficial for fluorescence-based detection, and the robust platform based on MEF is a promising tool for producing effective biosensors (Jeong et al. 2018).

Mei and Tang developed a biosensor based on multilayered hybrid nanocomposites for DNA detection using layer-by-layer (LbL) deposition technique. In such configuration, a layer of colloidal gold nanorods (AuNRs) was deposited on a glass substrate via solvent evaporation to be used as a nano-antenna for DNA detection and propagate the L-SPR for achieving MEF effect (Jeong et al. 2018; Mei and Tang 2016). Then another layer of fluorophore materials (i.e., dye) was loaded onto the AuNRs layers (see Fig. 12.20). A remarkable enhancement in the fluorescence intensity upon the attachment of DNA onto the AuNRs array chip as analyst is due to the MEF effect (Mei and Tang 2016).

In other configuration, which developed by Feng *et al.* based on using of functional materials such as polyelectrolyte to be a building block for fabrication of multilayered MEF-based biosensor. In such configuration, a layer of plasmonic nanostructures (i.e. AuNRs) was deposited onto a glass substrate using LbL technique, followed by loading of upconversion nanoparticles (i.e. lanthanide-doped NPs) as fluorophores. To achieve the MEF phenomena, the plasmonic AuNRs were separated from fluorescent lanthanide-doped NPs via deposition polyelectrolyte as a dielectric spacer as shown in Fig. 12.19b (Feng et al. 2015). Feng et al. reported that by modulation of the aspect ratio of AuNRs, the LSPR wavelength within the NIR region ~ 980 nm matches with the excitation wavelength of upconverted nanoparticles resulting in a remarkable fluorescence enhancement up to 22.6-fold with 8-nm spacer thickness. This proposed MEF-based biosensor configuration was a unique platform for bioimaging applications (Feng et al. 2015).

Moreover, a new type of biosensing platform, MEF-based biosensor, is developed by Ji and co-worker (Ji et al. 2016). In this biosensing system, Ag zigzag nanorod arrays were formed via LbL techniques using oblique angle deposition (see Fig. 12.19c) and were studied to determine whether it is suitable for MEF applications. By changing the fold number—the morphology of the Ag zigzag shape—a 14-fold and 28-fold enhancement factor is achieved for biotin-neutravidin, and the hybridization of two single-stranded oligonucleotides with 33-base detection was obtained (Ji et al. 2016). In addition, the limit of sensitivity was increased to 0.1 pM of targeted analyte.

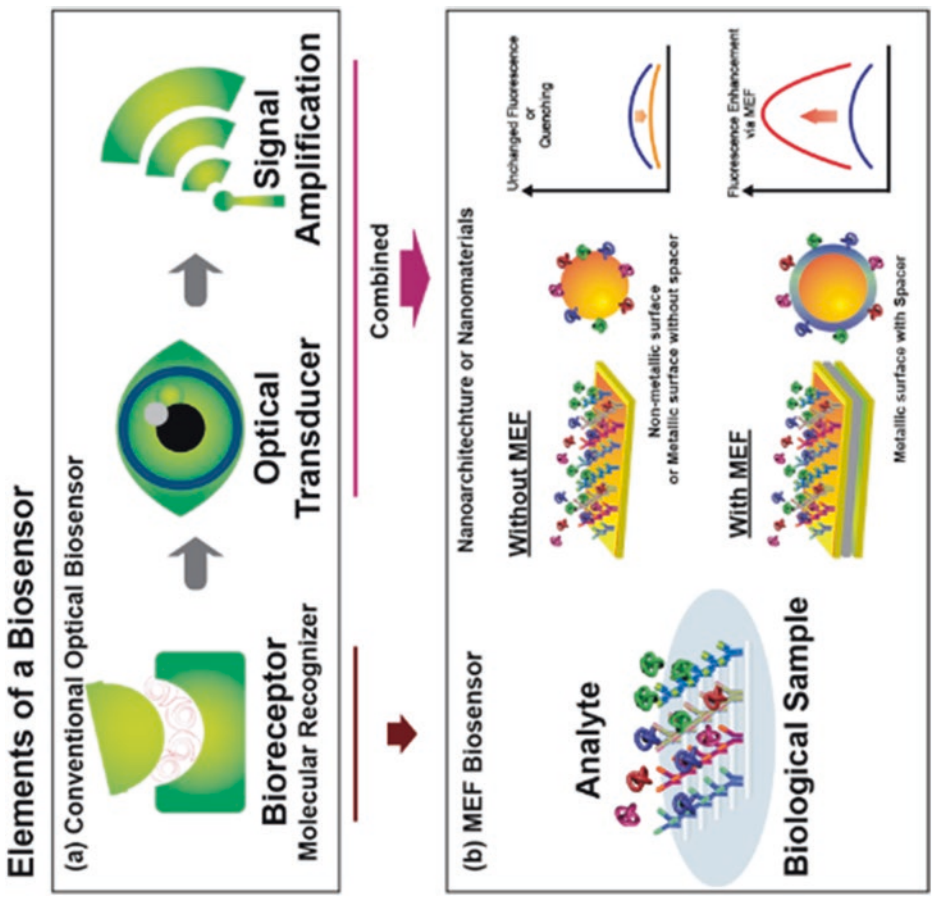

Fig. 12.18 The concept of optical biosensors, (**a**) conventional optical biosensor and (**b**) its correlation to MEF platforms for optical biosensors. (Reprinted with a copyright permission from Jeong et al. 2018)

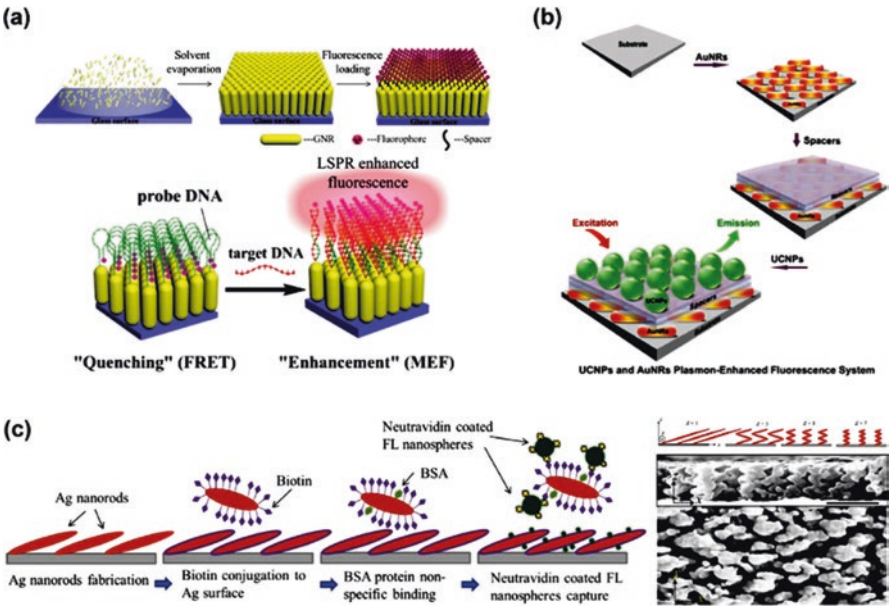

Fig. 12.19 Schematic diagram for fabrication MEF control via layer-by-layer (LbL) deposition. (**a**) The ordered gold nanorod (GNR) array chip for DNA detection upon hybridization. (**b**) Fluorescence enhancement of upconversion NPs (UCNPs) using polyelectrolyte multilayer deposition. (**c**) MEF of zigzag Ag nanorod arrays. (Reprinted with a copyright permission from Jeong et al. 2018, Mei and Tang 2016, Feng et al. 2015, Ji et al. 2016)

In other applications of biosensing, nanosystem based on detection of contaminants such as heavy metal (i.e., Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, and Cr^{3+} etc.…) has been demonstrated by Peng and co-workers (Peng et al. 2018). In such study, silica nanoparticles (SiO_2) are used to enhance the fluorescence properties of SGT1-SGT3 dyes and improve the detection sensitivity limits of SGTs-SiO_2 for heavy metal ions up to 1.81 and 0.0532 nM for Hg^{2+}, and Cd^{2+}, respectively.

Finally, Emam et al. developed a novel fluorescent and less toxic hybrid nanocomposites based on plasmonic/C-dots such as C-dots/PEI/Au and C-dots/PEI/Ag nanohybrids. These hybrid nanocomposites are prepared via chemical routes based on microwave irradiation method and physical conjugation of plasmonic nanostructures to PEI-coated C-dots (Emam et al. 2017b, 2018). A remarkable enhancement in the collective photo-physical properties (i.e., molar absorptivity and fluorescence quantum yield (FL-QY)). In addition, their approach allows the fabrication of engineered multi-modal hybrid nanocomposites based on MEF mechanism to be used in a wide range of applications such as chemical/biological sensing, probing, and theranostics (i.e. therapy and imaging), as shown in Fig. 12.20 (Emam et al. 2017b).

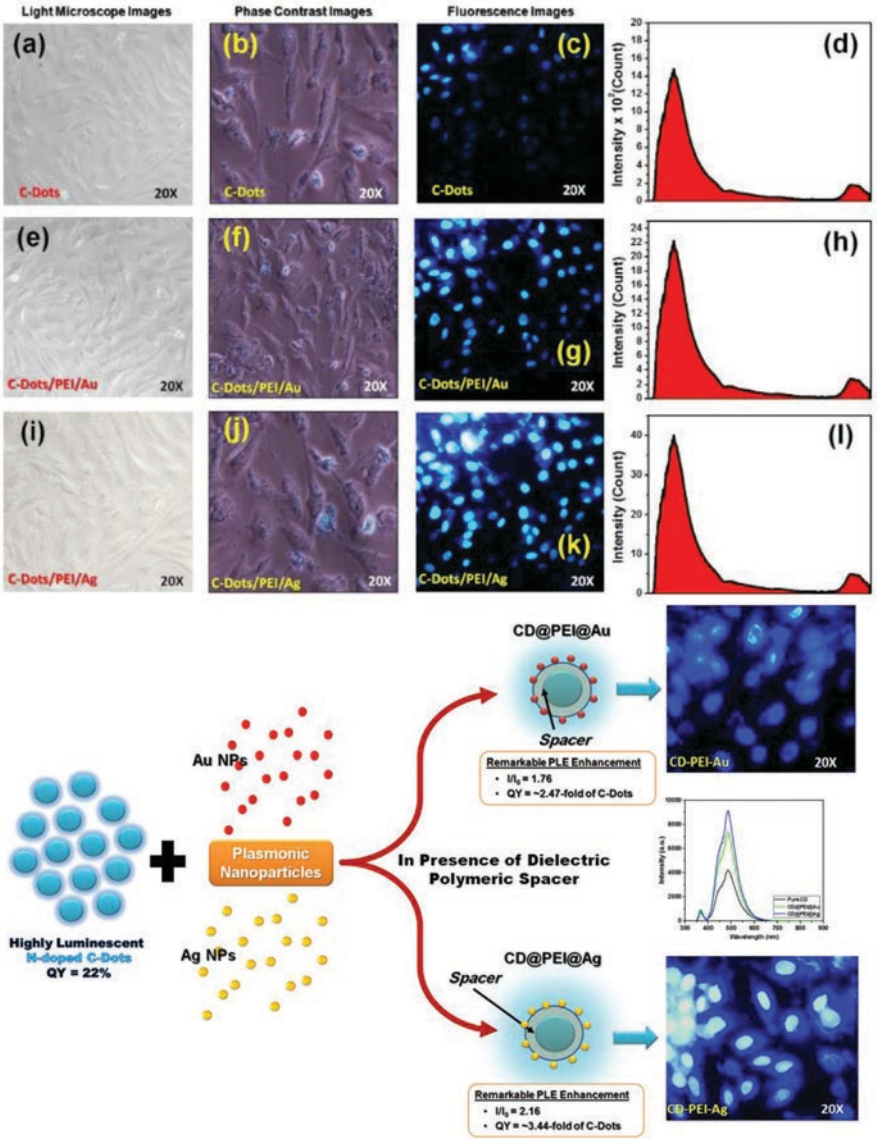

Fig. 12.20 Bright field (**a**, **e**, and **i**), phase contrast (**b**, **f**, and **j**), and fluorescence images (**c**, **g**, and **k**) of HepG-2 cells after 24-h incubation, and the intensity histogram (**d**, **h**, and **l**) of fluorescence images. (**a**, **b**, and **c**) for C-dots. (**e**, **f**, and **g**) for C-dots/PEI/Au nanohybrids. (**i**, **j**, and **k**) for C-dots/PEI/Ag nanohybrids. (Reprinted with a copyright permission from Emam et al. 2017b)

12.6 Conclusion

In conclusion, we introduced an overview about the optical properties of plasmonic nanomaterials and the parameters that affect the strength of the localized surface plasmon resonance (L-SPR), in addition to the required criteria to achieve successful metal-enhanced fluorescence (MEF) effect. Furthermore, in this chapter, we introduce a survey about our recent research works which was done regarding use of engineered hybrid nanocomposites to achieve MEF mechanism that opens new doors for multi-functional materials in so many applications such as chemical analysis, biosensing, biomedical imaging, and early diagnosis of cancers.

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