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Nanopharmaceuticals: Principles and Applications Vol. 3

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Nanopharmaceuticals: Principles and Applications Vol. 3

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Preface

Nanopharmaceuticals are at the forefront of advanced medicine and its applications are rapidly growing in the fields of drug delivery and diagnosis. The third volume of this book provides more insightful and updated information about the synthesis, safety, and toxicity of nanopharmaceuticals. This book also describes transport phenomenon, dental and dermal delivery of nanopharmaceuticals. A couple of chapters are devoted to the applications of nanopharmaceuticals on cancer therapy and diagnosis.

Chapter 1 emphasizes the safety issues of inorganic nanomaterials such as gold, silver, silica, copper, iron, and zinc nanoparticles. Toxicology profiling and cytotoxic studies of these nanoparticles were provided in this chapter.

Chapter 2 focuses on development and applications of nanoparticle-based biosensors for cancer detection. This chapter provides information on different types of biosensors capable of detecting biomarkers of cancer.

Chapter 3 is devoted to the novel approaches of nanotechnology towards advancements in dentistry. This chapter also focuses on different nanotools for dental ailments associated with bony as well as soft tissue defects.

Chapter 4 deals with the synthesis of both organic and inorganic nanostructures along with their characterization techniques and challenges faced during their development.

The first part of Chap. 5 deals with the basic concepts of electro spinning process and characterization techniques of electrospun nanofibers. The remaining part of the chapter covers drug-loaded nanofibers and recent applications of nanofibers in dermal drug delivery.

Chapter 6 provides the extensive information on nanomaterials for immobilization of enzymes and biomedical applications of enzyme immobilized nanocarriers. Challenges and critical opinions are also discussed at the end of the chapter.

Chapter 7 elucidates the toxicity of nanoparticles to different physiological systems and different methods to assess the toxicity of the nanoparticles for different organ systems using examples of in vivo systems.

Chapter 8 presents recent updates and insightful information on chemistry, synthesis, characterization, and applications of dendrimers.

Chapter 9 focuses on the main pathways of nanoparticles to penetrate and permeate the epithelial barrier and novel bio responsive delivery systems. Final part of the chapter deals with the toxicity of nanoparticles and its impact on organisms.

Chapter 10 provides information on advances in nano-theranostic materials. This chapter deals with nomenclature, bio-distribution, pharmacokinetics, surface functionalization, and application of nano-theranostics.

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About the Editors

Dr. Vinod Kumar Yata is an interdisciplinary researcher working at National Dairy Research Institute, Karnal, India. Previously, he worked as an Assistant Professor in Department of Biotechnology, Dr. B. R. Ambedkar National Institute of Technology Jalandhar, Punjab, India. Dr. Yata received his Ph.D. in Biotechnology from Indian Institute of Technology Guwahati. He specializes in interdisciplinary research which includes Nanotechnology, Microfluidics, Biotechnology, Cancer Biology, and Bioinformatics. Dr. Yata has developed a microfluidic device for the separation of live and motile spermatozoa from cattle semen samples. He opened up a new avenue for prodrug enzyme therapy by introducing nanocarriers for the delivery of non-mammalian prodrug activating enzymes. Dr. Yata elucidated the structural features and binding interactions of several bio molecules by *in silico* methods. He published several research papers in peer-reviewed international journals and presented papers in several international conferences.

Dr. Shivendu Ranjan has completed his B.Tech. and Ph.D. in Biotechnology from VIT University, Vellore, India, and has expertise in Nano(bio)technology. He was elected as a Fellow (FLS) of the oldest active biological society started in 1778, The Linnean Society (London), and he was an elected Fellow of Bose Scientific Society (FBSS). In 2018, he has been elected as Fellow of Indian Chemical Society (FICS) – a society founded in 1924. He has also been elected as Fellow (FIETA) of Indian Engineering Teachers Association. Currently, he is designated as a Senior Research Associate at Faculty of Engineering & Built Environment, University of Johannesburg, Johannesburg, South Africa. Recently he has been nominated as Strategic Head – Research & Development at Ennoble IP, Noida, India. He is also working as Visiting Faculty at the National Institute of Pharmaceutical Education and Research-R (NIPER-R), Lucknow. He is also designated as Vice President, Indian Chemical Society North Branch. Earlier, he has worked as Scientist at DST-Centre for Policy Research, Lucknow, supported by Ministry of Science and Technology, Govt. of India. He also worked as Head, Research & Technology Development at E-Spin Nanotech Pvt. Ltd., SIDBI Incubation Center, Indian Institute of Technology, Kanpur, India. He is also Advisor of many companies, e.g., Eckovation Solutions Pvt. Ltd. (IIT Delhi based start-up), Chaperon Biotech Pvt. Ltd (IIT Kanpur based start up), Kyntox Biotech India Pvt. Ltd., and Xcellogen Biotech Pvt. Ltd. Dr. Shivendu is also reviewer of Iran National Science Foundation (INSF), Tehran, Iran, and Jury at Venture Cup, Denmark, from the past 3 consecutive years. 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 is the Associate Editor of Environmental Chemistry Letters (Springer journal of 4.6 impact factor). He is serving as an editorial board member and referee for reputed international peer-reviewed journals. He has published several scientific articles as well as books and has h-index of 21. He has bagged several awards and recognition from different national as well as international organizations.

Dr. Nandita Dasgupta completed her B.Tech. and Ph.D. from VIT University, Vellore, India, and is Elected Fellow (FBSS) of Bose Science Society. She has major working experience in Micro/Nanoscience and is currently working as Assistant Professor at Department of Biotechnology, Institute of Engineering and Technology, Lucknow, India. Earlier, at LV Prasad Eye Institute, Bhubaneswar, India, she worked on Mesenchymal stem cell–derived exosomes for the treatment of uveitis. Dr. Dasgupta has exposure of working at universities, 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; and Indian Institute of Food Processing Technology (IIFPT), Thanjavur, 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 micro-vehicles for model drug molecules. Her areas of interest include Micro/Nanomaterial fabrication and its applications in various fields – medicine, food, environment, and agriculture biomedical.

Dr. Dasgupta has published 21 edited books and 1 authored book with Springer Switzerland. Dr. Dasgupta has authored many chapters and also published many scientific articles in international peer-reviewed journals. She is the associate editor of *Environmental Chemistry Letters* – a Springer journal of 3.2 impact factor – and also serving as editorial board member and referee for reputed international peer-reviewed journals. Dr. Dasgupta has received several awards and recognitions from different national and international organizations.

Dr. Eric Lichtfouse, Ph.D., 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 has founded the European Association of Chemistry and the Environment. He got 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
Inorganic Nanomaterials for Enhanced Therapeutic Safety

Sunaina Indermun, Mershen Govender, Pradeep Kumar, Yahya E. Choonara, and Viness Pillay

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Abstract Background/issues: Nanomaterials have been effectively and widely utilized in a variety of scientific disciplines to enhance biomedical applications. These nanomaterials are often organic or inorganic and often comprised of polymers or metal derivatives. The therapeutic safety of these often-toxic materials, however, is of paramount importance to ensure therapeutic safety. The safety of nanomaterials is therefore a widely undertaken research discipline evaluated both in vitro and in vivo. **Major advances:** This review provides for the currently undertaken research for the determination of therapeutic safety in inorganic nanomaterials. The importance of therapeutic safety, toxicity, and regulation of nanomaterials has been provided prior to the review of the respective nanomaterials. Specific focus has been given to metal-derived nanomaterials including gold, silver, silica, copper, iron, zinc, and titanium nanomaterials. Toxicology profiling and cytotoxicity studies of these nanomaterials have also been provided in addition to the in vivo studies that

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have been undertaken and the potential for alternative nanomaterial safety assessments.

Keywords Multifunctional nanomaterials · Toxicity · Physicochemical properties · Gold · Silver · Silica · Copper · Iron · Zinc · Titanium dioxide

1.1 Introduction

Exploited mainly for drug delivery applications, advances in the field of nanomaterials have been exponential. Offering the advantages of enhanced and efficacious delivery, nanomaterials improve the in vivo stability and solubility of active pharmaceutical agents (APIs) (Moghimi et al. 2001; Jia et al. 2013). Their nanosize correlates with many biological structures and organelles, making the nanomaterials appropriate for interactions at the submicron scale, thereby assisting in improving intracellular delivery, circulation, biodistribution, and the crossing of biological membranes (Singh and Lillard Jr 2009; Hassan et al. 2017).

Beyond the conventional approach of using nanomaterials as delivery vehicles, nanomaterials have also been designed and developed to confer individual functionality in the fields of energy generation, devices, therapeutics, biomedical applications, and chemical assays including medical imaging and antimicrobial coatings. The advent of nanomaterials has also led to the development of functionalized nanotechnology-based drug delivery systems that are able to diagnose, image, sense, and deliver therapeutics via conjugating moieties, such as aptamers, small molecules, and peptides (Lee et al. 2012).

Gold nanoparticles, silver nanoparticles, copper nanoparticles, magnetic nanoparticles, and mesoporous silica are some examples of inorganic nanocarriers which are amenable to functionalization and in addition can provide tracking capabilities (Subbiah et al. 2010). Although nanomaterials offer attractive advantages, physicochemical properties such as shape, size, surface charge, structure, composition, functionalization, and dissolution could significantly affect their cytotoxicity and therapeutic safety (Sharifi et al. 2012; Nel et al. 2013). Organic-based nanomaterials such as polymeric micelles, nanoparticles, and liposomes, primarily consisting of biocompatible amphiphilic copolymers, are well-known for their therapeutic safety (Bi et al. 2008; Oh et al. 2008). Contrasting studies have been conducted in the past where some authors have demonstrated that inorganic nanoparticles are in fact suitable for in vivo applications, whereas others have proved otherwise. Undoubtedly, the ability of nanomaterials to impart both action and interference at the cellular level renders many toxicity implications for such materials. This chapter will therefore detail the use, safety, and toxicity of inorganic nanomaterials with emphasis provided on the toxicology profiling and cytotoxicity studies of these nanomaterials in addition to the results of the in vivo studies that have been undertaken. Also provided for is the effectiveness of current safety profiling in addition to the potential for alternative nanomaterial safety assessments.

1.2 Mechanism of Toxicity

Nanoparticle toxicity is dependent on dose, route of administration, size, shape, lipophilicity, and exposure time and may interrupt the chemical and biological processes at various parts of the human anatomy such as at the molecular, cellular, and tissue levels (Johnston et al. 2010; Schrand et al. 2010; Wolfram et al. 2015). Interactions between these nanoparticles and body's biomolecules instantaneously occur upon delivery of the nanoparticles. This is due to the nanoparticles' high surface free energy which results in the biomolecules coating the nanoparticles, forming the protein corona (Monopoli et al. 2012; Wolfram et al. 2014, 2015). Consisting of both a hard and soft layer, the protein corona can drastically influence the nanoparticle size, shape, and charge, ultimately changing the amount of protein interactions (Fig. 1.1; Wolfram et al. 2014). Additionally, the endogenous biomolecules may also undergo structural and functional alterations as a result.

As mentioned, nanomaterial size, composition, and surface chemistry of nanomaterials are key determinants in their interactions with biological systems and their subsequent toxicity (Mirshafiee et al. 2017). These physicochemical properties may result in random membrane insertion, thereby leading to a cascade of signaling transductions that result in cytokine production and proinflammatory responses or eventual cell death. At the cellular level, peroxidative product accumulation, in vitro apoptosis, and cell antioxidant depletion can occur as a result of overproduction of reactive oxygen species (ROS) (Shang et al. 2014; Wang et al. 2016). Consequently, the redox state of the cell becomes imbalanced resulting in oxidative stress which has detrimental effects on the cells through protein, lipid, and DNA damage, resulting in cellular apoptosis and mutagenesis (Khanna et al. 2015). In order to ensure in vivo safety, well-defined methods to characterize and evaluate nanomaterials are required. Cellular homeostasis can be affected by inorganic nanomaterials, thus

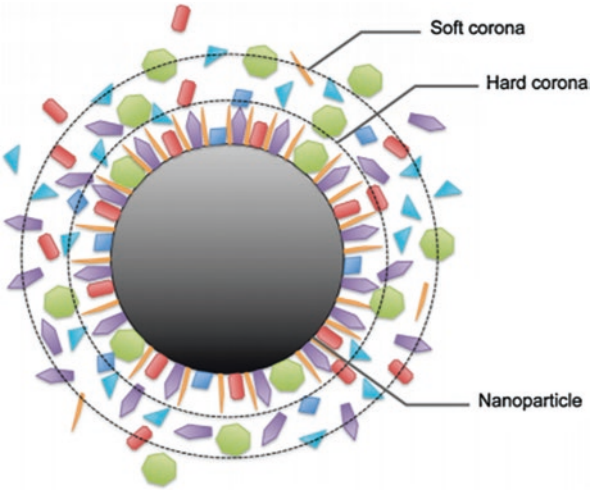

Fig. 1.1 Schematic representation of the current protein corona hypothesis. A hard and soft layer of proteins covers the surface of the nanoparticle. The proteins in the hard corona are more tightly associated with the particle surface, making them less dynamic than the proteins in the soft corona. (Reproduced from Wolfram et al. 2014, © 2014 Elsevier B.V)

allowing for a cascade of possible effects. Several mechanisms may result in such effects which are detailed in Fig. 1.2.

At the cellular level, nanoparticles may disrupt membrane integrity resulting in cellular leakage and disruption or destruction of cellular function. Lysosomal membrane dysfunction has been reported to be caused by polycation particles (Molinaro et al. 2013), zinc oxide (Cho et al. 2011), and titanium dioxide (Hamilton et al. 2009), resulting in endoplasmic reticulum stress, mitochondrial dysfunction, oxidative stress, and protein aggregation (Stern et al. 2012). Organ-related nanoparticle toxicity also occurs as a result of nanoparticle accumulation due to toxicity occurring at the molecular and cellular levels as well as through immunological responses.

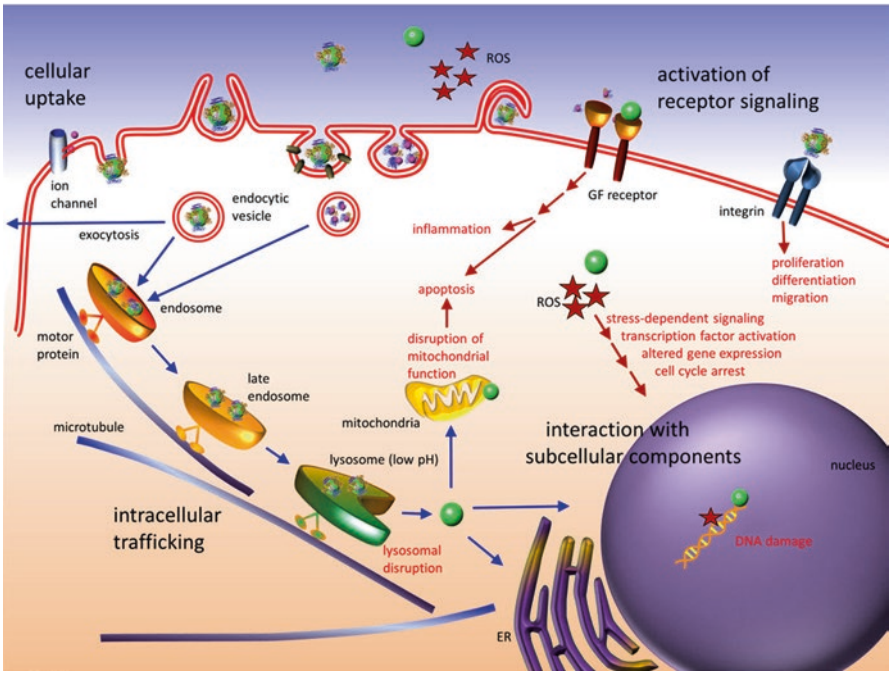

Fig. 1.2 Cytotoxic effects of nanoparticles. In the biological environment, nanoparticles may trigger the production of reactive oxygen species (ROS). Elevated ROS levels may lead to (i) activation of cellular stress-dependent signaling pathways, (ii) direct damage of subcellular organelles such as mitochondria, and (iii) DNA fragmentation in the nucleus, resulting in cell cycle arrest, apoptosis, and inflammatory response. Nanoparticles may interact with membrane-bound cellular receptors, e.g., growth factor (GF) receptors and integrins, inducing cellular phenotypes such as proliferation, apoptosis, differentiation, and migration. After internalization via endocytic pathways, nanoparticles are trafficked along the endolysosomal network within vesicles with the help of motor proteins and cytoskeletal structures. To access cytoplasmic or nuclear targets, nanoparticles must escape from the endolysosomal network and traverse through the crowded cytoplasm. (Reproduced from Shang et al. 2014 © Shang et al.; licensee BioMed Central Ltd. 2014, distributed under a CC-BY 2.0)

1.3 Inorganic Nanomaterials Used in Drug Delivery

1.3.1 Metallic Nanomaterials

Gold Nanoparticles

Possessing appreciable properties such as unique optical and electric properties and ease of functionalization with targeting moieties, drugs, and polymers using gold-thiol bonds, gold (Au) nanoparticles have been widely studied and employed in various applications such as drug delivery, photothermal delivery, and cellular and diagnostic imaging (Dreaden et al. 2012; Jia et al. 2013; Cheng et al. 2017). A study undertaken by Huo (2010) employed Au nanoparticle-based protein complex aggregation biomarker assays for the detection and diagnosis of cancer. Since these Au nanoparticles may be easily functionalized through simple bioconjugation techniques in a range of shapes and sizes, their cytotoxicity may thus be easily affected.

Below sizes of 4–5 nm in diameter, Au nanoparticles are catalytically active and may induce cytotoxicity (Falagan-Lotsch et al. 2016). Additionally, many toxicity studies have found Au nanoparticles with sizes greater than 4–5 nm in diameter to be nontoxic after acute exposure (Alkilany and Murphy 2010; Khlebtsov and Dykman 2011; Soenen et al. 2011). Particles of this size are considered mostly nontoxic to the mitochondrial cells; however, oxidative stress and mitochondrial damage can be incurred in cultured cells due to their high surface reactivity (Dreaden et al. 2012).

Au nanoparticles of 5 nm are generally used in nanomedicine and are presumed safe in drug delivery applications and photothermal therapy, with particles larger than 5 nm having the potential to result in cellular toxicity (Alkilany and Murphy 2010). Different cell type sensitivities and the use of high concentrations of Au nanoparticles may also attribute to other cases of acute toxicity (Patra et al. 2007; Mironava et al. 2010; Khlebtsov and Dykman 2011). After in vivo administration, Au nanoparticles have not been fully investigated for their long-term toxicological effects. Au nanoparticles have also been known to display an accumulation of degradation products as well as a reduced clearance of several months, possibly resulting in chronic toxicity (Khlebtsov and Dykman 2011; Kolosnjaj-Tabi et al. 2015). In addition, nephrotoxicity and erythrocytic cellular death have also been shown in vivo (Sereemaspun et al. 2008; Sopjani et al. 2008).

Studies have also shown that Au nanoparticle surface charge influences cellular uptake properties. The negatively charged cell surface residues have a higher affinity for cationic gold nanoparticles as compared to their anionic counterparts. Nevertheless, studies have demonstrated that the cationic nanoparticle surface charge can also result in an increased cytotoxicity in airway cells BEC and ASM and the ovarian cancer cells CP70, A2780 (Arvizo et al. 2010), and HeLa (Hauck et al. 2008) due to their altered surface properties associated with reduced particle size.

Additionally, due to their high biocompatibility and bioinert character, Au nanoparticles have also been extensively applied in diagnostic applications and gene delivery. However, for endothelial and epithelial applications, the presence of stabilizers such as sodium citrate residues on the Au nanoparticles was revealed to affect the proliferation and induce cytotoxicity in human alveolar type II (AT II)-like cells, human cerebral microvascular endothelial cells (hcMEC/D3), and human dermal microvascular endothelial cells (HDMEC). The study was carried out to determine if these effects were related to the varying degree of internalization of the Au nanoparticles, to surface sodium citrate on the Au nanoparticles, or to nanoparticle size. Differing uptake behaviors for citrate-stabilized Au nanoparticles were observed for the epithelial and endothelial cells. Concentration-dependent cytotoxicity was also observed after exposure to the Au nanoparticles (Fig. 1.3). It was demonstrated that the lower the degree of purification, the less cell viability and proliferation occurred concluding that the safety of Au nanoparticles may be enhanced with the abridged addition of sodium citrate (Freese et al. 2012).

Falagan-Lotsch and coworkers (2016) investigated the long-term in vitro effect on human dermal fibroblasts of two shapes of Au nanoparticles. The study was conducted on both nanorods and nanospheres under both chronic and nonchronic conditions. It was determined that the oxidative stress and inflammation gene expression could be modified with a subcytotoxic dose of Au nanoparticles, with the effect lasting over 20 weeks. The results indicated that the cell stress response is not reversible over time upon removal of the nanoparticles after acute exposure and that the cells can adaptively respond to chronic, low-level nanoparticle insults (Falagan-Lotsch et al. 2016). Interestingly, in the study undertaken by Falagan-Lotsch et al. (2016), the surface chemistry of polyethylene glycol was found to be not as benign as is generally assumed.

These studies investigating the toxicological effects of Au nanoparticles therefore suggest that long-term studies are warranted, rather than their acute counterparts, to elucidate better, safety profiles of gold nanomaterials.

Silver Nanoparticles

Used in various antimicrobial applications, silver (Ag) nanoparticles have been implicated in major health concerns due to their toxicological impacts on various organs (Mirshafiee et al. 2017). A notable effect of chronic silver exposure is argyria in humans (Wijnhoven et al. 2009). Ag nanoparticles release toxic silver ions following particle dissolution resulting in significant cytotoxicity via ROS generation (Wang et al. 2014; Zhornik et al. 2014; Osborne et al. 2015; Zhang et al. 2015).

Furthermore, these Ag nanoparticles may migrate to the brain, lungs, kidneys, liver, and spleen following detachment from colloidal silver wound dressings (Ahamed et al. 2010). Peripheral multiorgan inflammation caused by Ag nanoparticles was demonstrated by Guo and coworkers (2016). Focusing on interendothelial junctions, the mechanisms of action of Ag nanoparticles and silver nitrate ($AgNO_3$) were compared employing primary human umbilical vein endothelial

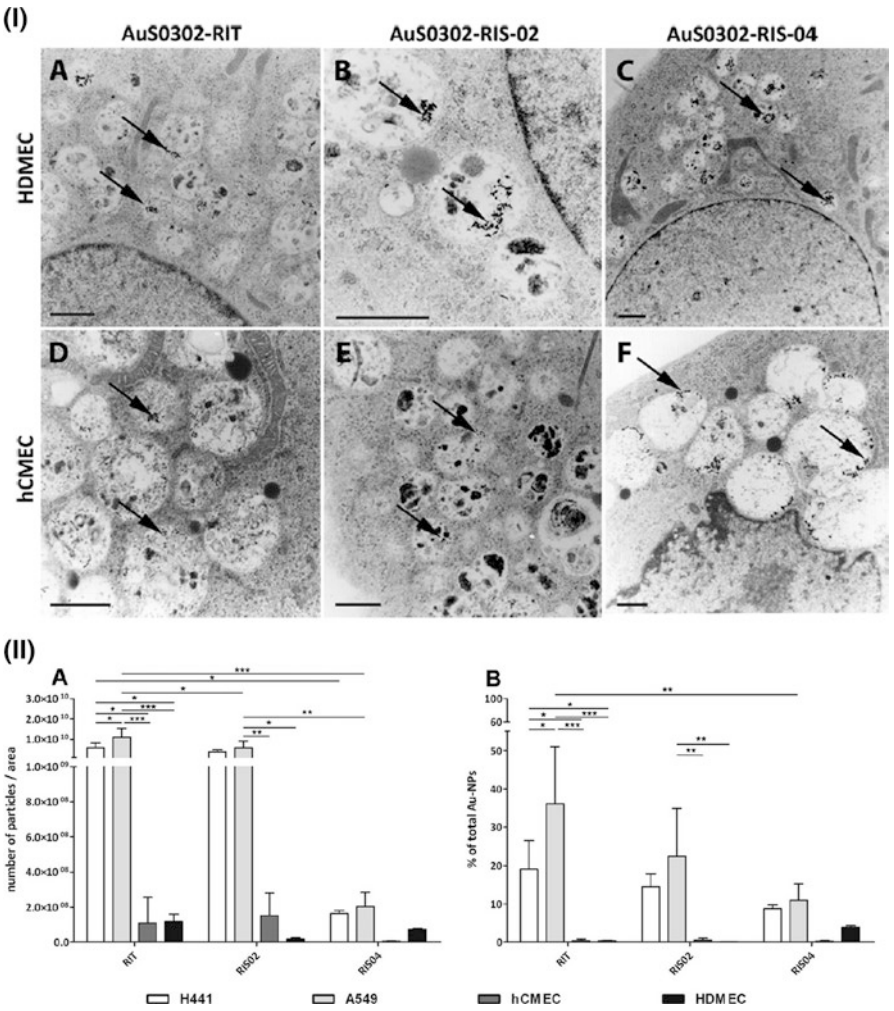

Fig. 1.3 **(I)** Internalization of gold nanoparticles in HDMEC and hCMEC/D3 analyzed by transmission electron microscopy. HDMEC (**a**–**c**) and hCMEC/D3 (**d**–**f**) were incubated with 300 μM gold nanoparticles for 24 h. After exposure, cells were extensively washed, fixed with paraformaldehyde, and examined by transmission electron microscopy (TEM). AuS0302-RIT, AuS0302-RIS02, and AuS0302-RIS04 were found in intracellular vesicles which were mostly located in the perinuclear region. The arrow heads indicate the gold nanoparticles within the vesicles. Scale bar: 1 μm. **(II)** Quantification of internalized gold nanoparticles in endothelial and epithelial cells by ICP-AES. Both epithelial cells (H441 and A549) and endothelial cells (HDMEC and hCMEC/D3) were incubated with 50 μM gold nanoparticles at 37 °C for 24 h. Cells were extensively washed, lysed by aqua regia (3:1 hydrochloric acid/nitric acid), and analyzed for gold concentration by ICP-AES. In (**a**) the total number of particles per area was calculated, while in (**b**) the percentage uptake of particles into cells, as a function of the total amount applied, was determined. (Reproduced with permission from Freese et al. 2012 © Freese et al.; licensee BioMed Central Ltd. 2012, distributed under a CC-BY 2.0)

cells (HUVEC). It was shown that endothelial endocytosis was primarily due to Ag nanoparticles as opposed to $AgNO_3$. This study determined increased intracellular ROS and VE-cadherin downregulation resulting in the disruption of the integrity of the endothelial layer between the endothelial cells caused by Ag nanoparticles which interestingly could be remedied by N-acetylcysteine (Fig. 1.4). However, $AgNO_3$ (>20 μg/mL) resulted in direct cell death without ROS induction at lower concentrations. Notably, peripheral inflammation was induced in the liver, lungs, and kidneys from Ag nanoparticle release with the severity increasing in relation to the diameter of the Ag nanoparticles used.

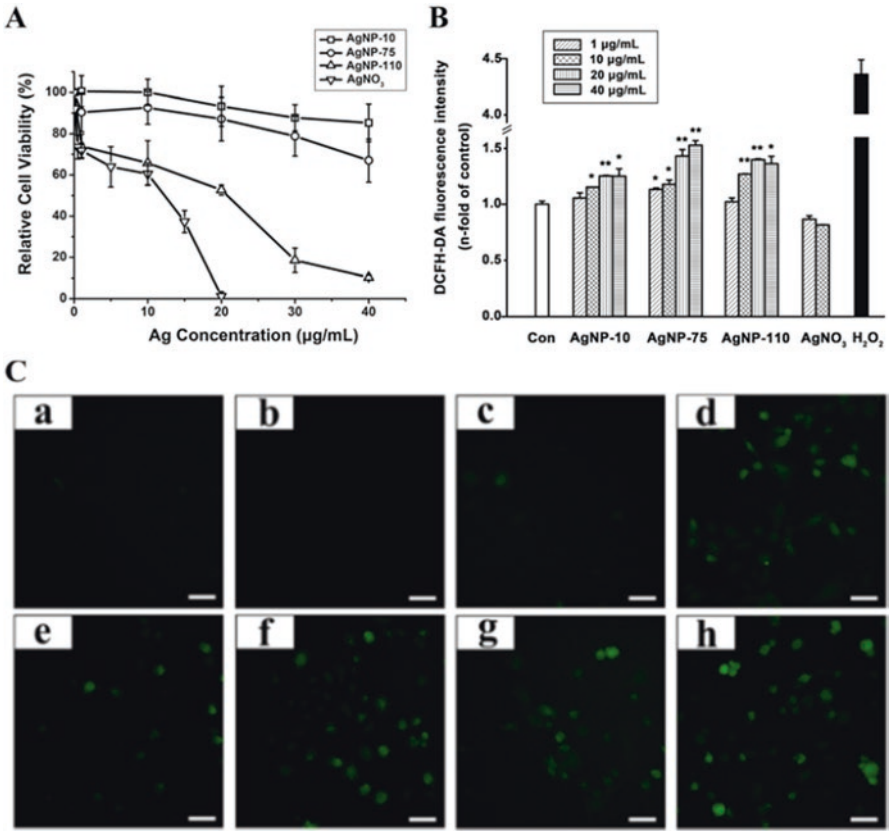

Fig. 1.4 The viability and intracellular ROS of cells exposed with Ag nanoparticles or $AgNO_3$: (A) Cell viability from CCK-8 assay. (B) The intracellular ROS level caused by Ag nanoparticle or $AgNO_3$ exposure for 1 h. The H_2O_2 group was set as the positive control. The * represents significant difference between control group and Ag nanoparticle-75-treated group (*: $p < 0.05$, **: $p < 0.01$). (C) Representative fluorescence images of cells stained by DCFH-DA, in which (a) control group, (b and c) cells incubated with $AgNO_3$ at 1 μg/mL and 10 μg/mL of Ag, (d) cells exposed to 7.5 mg/mL H_2O_2, (e–h) cells treated with 1, 10, 20, and 40 μg/mL Ag nanoparticle-75. The scale bar represents 50 μm. Ag nanoparticle toxicity depends on surface chemistry and particle size. (Reproduced from Guo et al. 2016 © Guo et al. 2016, distributed under a CC-BY 4.0 license)

Studies conducted by Wang and coworkers (2014) determined that polyvinylpyrrolidone- and citrate-coated Ag nanoparticles (20 nm) cause more oxidative stress and cellular toxicity than larger particles (110 nm) due to their bioavailability and higher rate of dissolution. The pulmonary impact was assessed in vivo, where the large Ag nanoparticles were shown to cause more significant subchronic lung injury at 21 days due to a slower dissolution rate, whereas the smaller silver particles (20 nm) induced higher acute lung inflammation. This study has demonstrated the size and dissolution effects on biopersistence and lung inflammation.

Copper Nanoparticles

A vital micronutrient in all tissue, copper (Cu), is mandatory for various cellular functions: cellular pigment formation, neurotransmitter biosynthesis, connective tissue strength, respiration, and peptide amidation (Araya et al. 2003; Desai and Kaler 2008; Ude et al. 2017). The preservation in Cu homeostasis is essential in preventing possible neurological diseases such as Huntington's and Alzheimer's disease (Kaler 1998; Gaggelli et al. 2006). Used in an array of products such as cosmetics, textiles, inks, antimicrobials, and food contact materials, it is pertinent that copper-containing nanomaterials be evaluated for possible toxicity.

Of the metallic nanomaterials, copper oxide nanomaterials (42 nm) were deemed to be the most cytotoxic in comparison to iron complexes ($CuZnFe_2O_4$, Fe_3O_4, and Fe_2O_3), titanium oxide (TiO_2), and zinc oxide (ZnO). They were seen to exhibit the most DNA damage to the A459 human lung epithelial cell line (Karlsson et al. 2008). However, studies on the toxicity of ingested copper oxide (CuO) nanomaterials are few. A recent study by Ude and coworkers (2017) has exploited the cytotoxic impact of CuO nanomaterials on intestinal epithelial cells (Fig. 1.5). Employing undifferentiated Caco-2 intestinal cells, CuO nanomaterials, and $CuSO_4$, the study evaluated the toxicity comparability of both CuO nanomaterials and $CuSO_4$ in vitro, suggesting particle- and ion-mediated mechanism effects due to the less soluble CuO nanomaterial. The CuO nanomaterials displayed concentration-dependent decreases in undifferentiated cell viability, yet no discernable difference was seen between the cytotoxicity of CuO nanomaterials and $CuSO_4$. Additionally, important for risk assessment, CuO nanomaterials were proven to be no more potent than the $CuSO_4$.

An interesting study by Murugan and coworkers (2017) investigated the function of geometrical structure of copper nanoparticles (Fig. 1.6). Nanoparticles were synthesized with a dual functionality comprising the ability to induce cytotoxicity on proliferating cells as well as geometric attributes for enhanced cellular uptake. Extensive cellular internalization studies were conducted using HeLa and NHEK cell models. The primary toxicity factor was attributed to the effect of the nanogeometry of the copper nanoparticles. Cell viability was also observed to be dose dependent. Interestingly, results displayed a significant difference in toxicity between the two cell lines and the geometrical nanoparticles. On the NHEK cell line, cell viability was observed to be 33.33% at the highest Cu nanoparticle

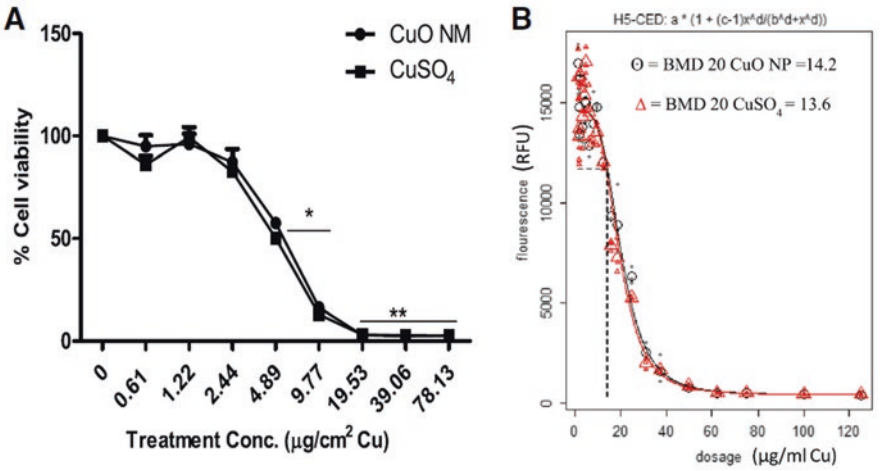

Fig. 1.5 Cytotoxicity of CuO nanomaterials and $CuSO_4$ to undifferentiated Caco-2 cells. Viability of undifferentiated Caco-2 cells was assessed using the Alamar Blue assay following exposure of cells to cell culture medium (control), CuO nanomaterials, or $CuSO_4$ at concentrations ranging from 0.61 to 78.13 µg/cm^2 Cu for 24 hours. (**a**) Viability of Caco-2 cells following CuO nanomaterial or $CuSO_4$ exposure expressed as a % of the control. (**b**) Determination of 20% benchmark dose (BMD 20) in µg/ml following exposure of undifferentiated Caco-2 cells to CuO nanomaterials or $CuSO_4$ exposure. Data was analyzed using Proast 38.9 software to obtain the BMD 20. Data are expressed in mean ± SEM (n = 3), and * represents significance compared to control at $P < 0.05$. (Reproduced from Ude et al. 2017 © Ude et al. 2017, distributed under a CC-BY 4.0 license)

concentration with the median lethal concentration (LC_{50}) values occurring at approximately 12.5 µg/ml and 25 µg/ml, respectively, for the NHEK and HeLa cell lines.

Iron Nanoparticles

There are currently several iron nanoparticles approved by the FDA for therapeutics and imaging purposes (Hassan et al. 2017). Iron nanoparticles may be functionalized for different therapies but have primarily been used for targeted drug delivery, protein separation, magnetic hyperthermia, and MRI (Mahmoudi and Shokrgozar 2012; Schladt et al. 2012). Superparamagnetic iron oxide nanoparticles (SPIONs) display lower toxicity in comparison to other contrast agents (Mirshafiee et al. 2017). AAs an example, in hepatic imaging, following administration, the SPION particles are expected to be phagocytosed by the hepatic Kupffer cells (Wang 2011). Since lower uptake is expected in the diseased hepatic region, a concentrated signal will be generated by the SPIONs to more aptly identify lesions. Following intracellular uptake, SPIONs dissolve into a nonsuperparamagnetic form of iron ions which is further hepatically metabolized and subsequently excreted via kidneys or utilized in red blood cell formation (Weissleder et al. 1989).

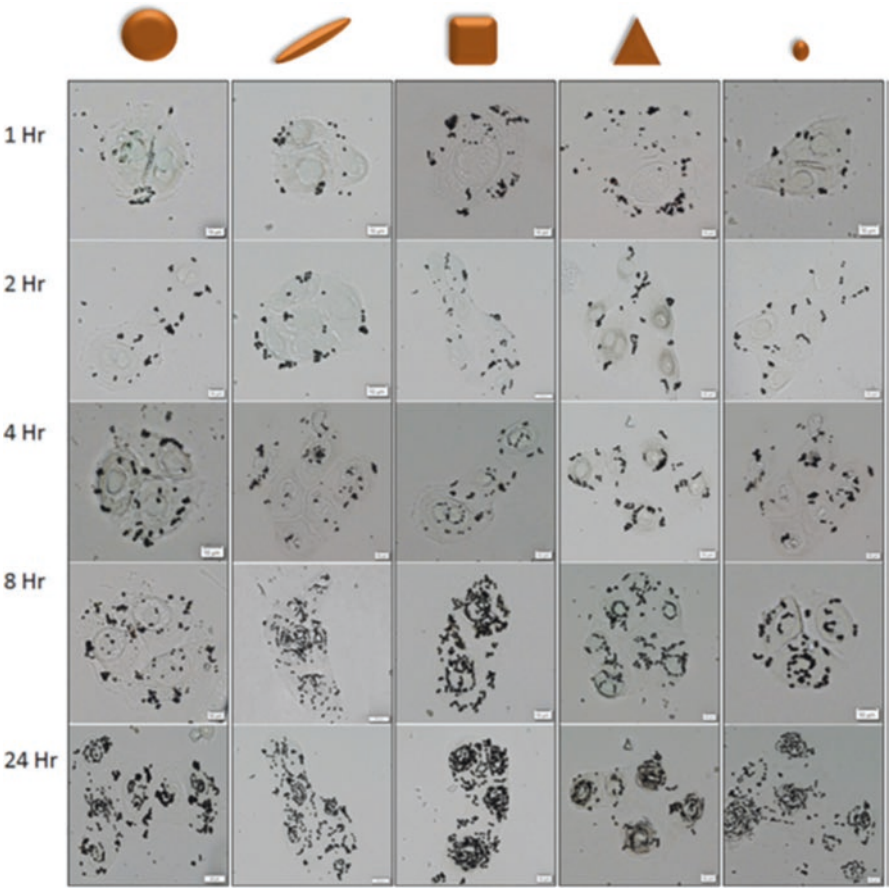

Fig. 1.6 Phase contrast images of geometric Cu nanoparticle internalization over a 24 hour incubation period. (Reproduced with permission from Murugan et al. 2017, © 2017 Elsevier B.V)

Lunov and coworkers (2010) purport increased ROS production by SPIONs that eventually lead to Kupffer cell apoptosis via the ferrous ions (Fe^{2+}) released via the Fenton reaction. This, in turn, reacts with mitochondrial hydrogen peroxide and oxygen ultimately inducing oxidative stress. Moreover, with frequent administration or prolonged treatment, SPION accumulation can result in elevated lipid metabolism, disruption of iron homeostasis, as well as liver dysfunction. Thus, to combat or possibly reduce such adversities associated with SPION use, surface coatings (e.g., dextran and silicon) have been applied to improve biocompatibility but do not address iron accumulation issues in the body (Mirshafiee et al. 2017).

Interestingly, DeLoid and coworkers (2017) reported on the evaluation of nanoparticle biokinetics and toxicity using an iron oxide (Fe_2O_3) and corn oil in phosphate buffer emulsion. This nanoenabled food was passed through a GIT simulator. The study determined the influence of food and GIT components on

nanoparticle biokinetics, transport, and toxicological profile. Fe_2O_3 was found to be nontoxic with the Fe_2O_3 translocation after 4 h being <1% and ~2% for digesta with and without serum, respectively. Results from this study suggest the alteration of nanomaterial biokinetics by serum proteins, raising concerns about the neglect of such food–GIT interactions.

Producing a multifunctional nanomaterial, Zhang and coworkers (2012) exploited the use of SiO_2-coated magnetic Fe_3O_4 nanoparticles based on the premise of avoiding iron leaching in acidic biological environments. Addressing chemotherapeutic applications, these nanorattles were produced through an ion exchange process and consisted of hydrophilic, rare-earth-doped $NaYF_4$ shells. Displaying appreciable drug-loading capacity and excellent water dispersibility, this system allowed for both upconversion magnetic and luminescent properties and was found to shrink tumors in vivo by simultaneously delivering doxorubicin (DOX) and enhancing tumor targeting (Fig. 1.7).

Zinc Nanoparticles

Zinc oxide (ZnO) nanoparticles have been utilized in cosmetics as well as in sunscreen lotions due to their UV-blocking ability. These nanoparticles have been purported to induce cytotoxicity through ROS generation, affecting endothelial cell function and causing possible damage to intracellular organelles (Abukabda et al. 2016).

Kura and coworkers (2015) evaluated for acute oral toxicity in Sprague Dawley rats using a zinc–aluminum–LDH–levodopa nanocomposite (ZAL) and zinc–aluminum nanocomposite (ZA). Employing a layered double hydroxide (LDH) nanocarrier system, the results suggested that acute toxicity in the rats was not induced by ZAL and ZA at 2000 mg/kg body weight, suggesting safe, acute, oral administration of zinc–aluminum.

In another study by Kolesnikova and coworkers (2011), nanocomposite microcapsules with zinc oxide nanoparticles in their shells were fabricated using layer-by-layer assembly. Constituent components of the microcapsule shell included both poly(allylamine hydrochloride) solution (PAH) and poly(sodium styrene sulfonate) solution (PSS). Results indicated that the acute toxicity effect in comparison with the constituent components was significantly decreased for the suspension of the microcapsules.

Fig. 1.7 (continued) H22 xenograft tumor were injected with DOX-loaded MUC-F-NR (1 mg/kg) and subjected (+MF) or not subjected (−MF) to the magnetic field for 1 h. At 24 h postinjection, mice were imaged in vivo. (**c**) The luminescence signal was measured from the whole tumor in vivo and ex vivo. (**d**) Tumor volume changes of saline-treated mice compared to mice treated with MUC-F-NR, DOX, and DOX-loaded MUC-F-NR over 21 days in the absence and presence of magnetic field. Data show mean ± SD (n = 5, *p e 0.05). (Adapted with permission from Zhang et al. 2012 ©, 2011 American Chemical Society)

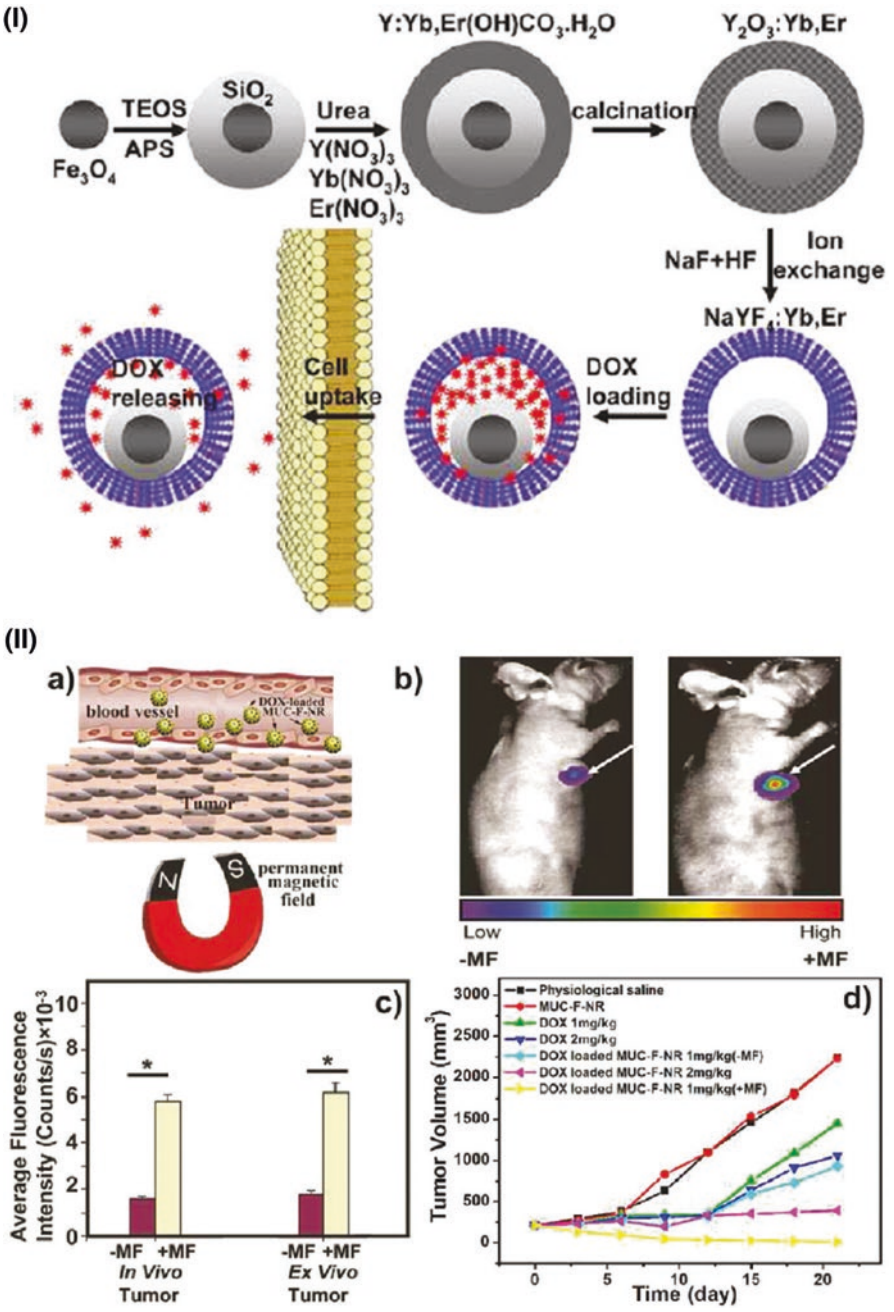

Fig. 1.7 **(I)** Synthetic procedure for the drug-loaded Fe_3O_4@SiO_2@α-NaYF/Yb, Er nanorattles (DOX-MUC-F-NR). **(II)** (**a**) Schematic illustration of targeting of DOX-loaded multifunctional drug carrier to tumor cells assisted by an externally applied magnetic field (MF). (**b**) Tumor location as defined by MUC-F-NR intensity increases with 1 h magnetic field treatment. Mice bearing-

(continued)

Titanium Dioxide

The literature available on titanium dioxide TiO_2 is vast as this metal oxide nanoparticle is widely exploited. TiO_2 is a white pigment with a very high refractive index and is thus commonly included in inks, paints, papers, pharmaceuticals, medicines, food products, and toothpaste and along with zinc oxides in sunscreens and cosmetics (Shi et al. 2013). Several investigations have focused on the dermal penetration and toxicity of TiO_2. In a study by Schulz and coworkers (2002), particle size, coating, and shape were evaluated for its effect on TiO_2 skin penetration (4 mg/cm^2). Several TiO_2-coated sunscreens including aluminum oxide (Al_2O_3), silica (SiO_2) (10–15 nm), and trimethyloctylsilane (20 nm) were exposed topically to human skin for 6 h. Results indicated that TiO_2 did not penetrate the skin. Similarly, a study conducted by Mavon and coworkers (2007) determined TiO_2 (20 nm) distribution both in vitro and in vivo. Five hours post direct topical application (2 mg/cm^2), tape stripping was used to determine the dermal penetration of TiO_2. It was found that there was minimal TiO_2 distribution within the epidermis.

Concern over the potential cytotoxicity of TiO_2 nanoparticles stems mainly from that of the pulmonary adverse effects of TiO_2 with many dermal TiO_2 distribution studies concluding that TiO_2 nanoparticles are not systemically available to a significant extent after dermal exposure. Originally emanating from studies by Ferin and coworkers (1990, 1992) and Oberdorster and coworkers (1990), ultrafine TiO_2 was demonstrated to enhance pulmonary inflammation and particle retention and translocation. These studies have led to the reassessment of TiO_2 as a negative control in pulmonary toxicology studies when assessing the toxicity of pathogenic particulates such as alpha-quartz (Johnston et al. 2009). The limit for fine particles in the air is 50 μg/m^3 for an average human of 70 kg (Simko and Mattsson 2010). Acute toxicity information for TiO_2 nanoparticles in humans, however, is currently lacking (Shi et al. 2013).

1.3.2 Nonmetallic Nanomaterials

Silica-Derived Nanoparticles

Silica (Si) nanoparticles offer exemplary characteristics such as rapid in vivo degradation, chemical conjugation-mediated camouflage (Parodi et al. 2013), metal incorporation for theragnostic applications (Lee et al. 2011), and regulation of pore sizes (2–10 nm) for drug encapsulation (Gao et al. 2011). Cutaneous absorption of this metalloid nanomaterial through the skin often simultaneously occurs with exposure to other environmental allergens as well as other chemical compounds, yet these potential associated hazards have not been thoroughly investigated (Li et al. 2008).

A study conducted by Hirai and coworkers (2015) investigated the concurrent topical application of amorphous silica nanoparticles and mite extract on human

atopic dermatitis and allergic sensitization in NC/Nga mice. Low-level production of allergen-specific IgGs was observed after concurrent cutaneous exposure of the nanoparticles and allergens. Additionally, following exposure to the allergen–silica nanoparticle agglomerates, low-level IgG production was induced in the mice, but this was not observed when exposed to well-dispersed nanoparticles or nanoparticles applied separately from the allergen. This research conducted suggests that the allergen-specific immune response is not directly affected by silica nanoparticles. However, it should be noted that the Si nanoparticles led to a key risk factor of atopic allergies in humans as well as a low IgG/IgE ratio, when present in allergen-adsorbed agglomerates.

Mesoporous silica nanoparticles, the advancement of silica (Si) nanoparticles, have been utilized to overcome the issues associated with biocompatibility, degradability, and drug release rates related to metallic or other inorganic nanomaterials (Hassan et al. 2017). Mesoporous silica nanoparticles have since been functionalized to regulate biodistribution and reduce systemic toxicity. "Cloaking" of the mesoporous silica nanoparticles, i.e., coating with leucocyte membranes, has been found to reduce cytotoxicity while enhancing the delivery of doxorubicin in vivo (Parodi et al. 2013). Kim and coworkers (2016) investigated functionalized poly(ethylene glycol)-coated (PEGylated) near-infrared (NIR) fluorescent silica nanoparticles that were functionalized with melanoma-targeting peptides. This hybrid organosilica particle demonstrated the ability to induce cell death and ferroptosis as well as the inhibition of tumor growth and tumor regression with high-dose particle delivery.

Nanoclay-Derived Delivery

The implementation of nanoclays in industrial and commercial commodities has increased exponentially over the years. In the pharmaceutical industry, nanoclay–polymer-based composites have allowed for improved mechanical strength and reinforcement properties. Due to their fine and nanoparticulate nature, nanoclays have been investigated for toxic effects on lung health (Wagner et al. 2017). Studies have shown that nanoclays, on a cellular level, display mitochondrial damage, ROS generation, and membrane and cellular damage effects (Wagner et al. 2017). Most clays have been deemed as non-toxic and have thus been extensivley studied for their biomedical applications such as drug delivery, preparation of scaffolds and tissue engineering. Studies by Wang and Tong (2008) and Michael and coworkers (2016) have investigated the effects of nanoclays, for bone cement applications. Results of both studies have determined increased bioactivity and mechanical properties. Yang and coworkers (2017) developed semi-IPN sericin/poly(NIPAm/LMSH) (HSP) nanocomposite hydrogels for wound healing applications. The nanocomposites were shown to result in almost complete recovery by day 13 of the study.

1.4 Safety of Nanotheragnostic Agents

Theragnostics synergistically employs the use of both diagnostics and therapeutics culminating into more safe and efficacious personalized disease management. Commonly used in ultrasound, single photon emission computed tomography (SPECT), positron emission tomography (PET), and magnetic resonance imaging (MRI), theragnostic nanoparticles can be categorized into therapeutic payload, payload carrier, signal emitter, and targeting ligand (Fang and Zhang 2010). Theragnostic nanoparticles possess qualities that allow for sufficient, targeted drug delivery; specific, rapid, and selective targeting; reporting of biochemical and morphological disease characteristics; and rapid, efficient clearance without the formation of toxic by-products (Jokerst and Gambhir 2011; Chen et al. 2014). Though studies on theragnostic nanomaterial toxicity are limited, many studies have employed theragnostic approaches with functionalized engineered magnetic nanoparticles for MRI-guided therapeutic cell replacement and MRI-assisted diagnosis and surgeries (Shubayev et al. 2009).

Notably, the superior superparamagnetic theragnostic qualities of iron oxide engineered magnetic nanoparticles have been safely and effectively used in MRI with many dextran-coated nanoformulations being approved for clinical use as MRI contrast agents, i.e., ferumoxtran, ferucarbotran, and ferumoxides (Shubayev et al. 2009). Even though iron deposits have been associated with many neurodegenerative diseases such as Alzheimer's disease, multiple sclerosis, Parkinson's disease, and Huntington's disease, iron oxide engineered magnetic nanoparticles were shown in studies to be the safest of the metal oxide nanoparticles only producing cytotoxic effect at 100 μg/ml or higher (Hussain et al. 2005; Jeng and Swanson 2006; Gojova et al. 2007). Dextran-coated magnetite nanoparticles were found to exert cytotoxic effects at 400 mg/kg in rats (Lacava et al. 1999; Lacava et al. 2004).

1.5 Nanomaterial Hazard Assessment

Relative to the toxicity potential of nanomaterials, risk assessments in this subject matter have been exponential (OECD 2005, 2007, 2010a, b, 2012a, b, c, 2014, 2015, 2016a, b, c, d, e, f). Depending on particular nanomaterial characteristics such as various biological indicators, structure activity, physicochemical, in vitro test results, or in vivo test results, predictive toxicological modeling is achieved (Schulte et al. 2018). In vivo exposure systems have been extensively utilized to address these concerns and have been instrumental in addressing the potential safety concerns regarding nanomaterials. Since the ethical support of the replacement of animals with more human-relevant alternatives, the principle of the 3Rs – Replacement, Reduction, and Refinement – has become an increasing mandate (Tornqvist et al. 2014; Drasler et al. 2017). A stepwise approach to categorize the need for validated in vivo studies based on positive effects of in vitro cell experiments involving

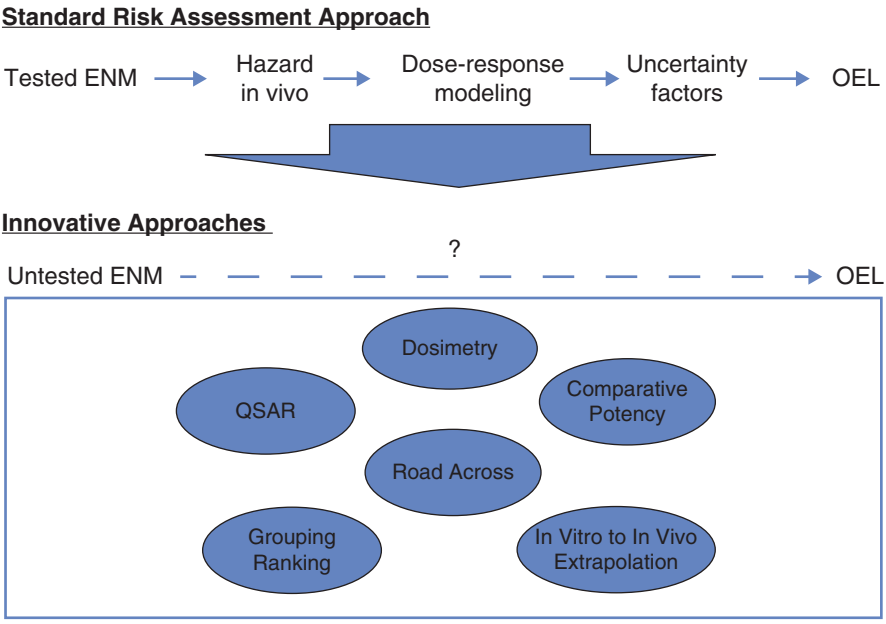

Fig. 1.8 Frontier of risk assessment for developing occupational exposure limits for engineered nanomaterials. Abbreviations: *ENM* engineered nanomaterial, *OEL* occupational exposure limit, *QSAR* quantitative structure–activity relationship. (Reproduced with permission from Schulte et al. 2018, © Elsevier Inc.)

nanomaterials exists and is highlighted in Fig. 1.8. However, hurdles such as in vivo behavior of the nanomaterials and the ability to create additional functionality through sophisticated fabrication methods are warranted (Cheng et al. 2012). Regulatory bodies, consumer, and society expectations about their safety are increasing, and as with all developing technologies, it is pertinent to identify possible hazards and develop risk assessment and management approaches (Drasler et al. 2017; Meldrum et al. 2017). Hansen (2010) and Breggin and coworkers (2009) sufficiently provide for an overview of national and international initiatives to regulate nanomaterials.

1.6 Conclusion

Nanomaterials have been extensively utilized for their enhanced biomedical properties. Information and data on the safety of these materials in the physiological environment is however essential prior to the use in the treatment or prevention of any physiological conditions. This review has provided an extensive account on the studies that have been undertaken on gold, silver, copper, iron, zinc, and titanium as well as on silica and nanoclay nanomaterials with focus given on their therapeutic

safety profiles as well as the cytotoxic effects due to varying particle size and concentration. All evaluated nanomaterials have been noted to be inherently cytotoxic; however, research into these respective materials has aimed to increase their biocompatibility through modification of particle size, shape, and charge as well as through the use of metal derivatives with increased safety profiles. The alternatives to cytotoxicity and in vivo studies with the aim to minimize the use of animal models in the therapeutic safety studies have also been provided to highlight the advancements in therapeutic safety analyses. Therapeutic safety analysis of nanomaterials is therefore a vital tool to ensure the effective use of potentially toxic materials in the treatment and prevention of physiological conditions.

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Chapter 2
Nanoparticles Application for Cancer Diagnosis

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Abstract Biosensor as a diagnostic platform could provide the opportunity for cancer detection at the molecular levels. Due to the complexity of the cancer biology in patient, development of versatile sensors for molecular diagnosis of cancer is a challenging issue. Various molecular biomarkers of cancers could be implemented as a target for preparation of diagnostic biosensors for cancer detection. In this regard, some specific biological properties of cancer cells comprising genetic markers, protein expression, gene expression, and posttranslational modifications of proteins could be used as molecular signature of cancer which plays a crucial role in development of biosensors for cancer diagnostic purposes. Different methodologies *based* on *nanoparticles* (NPs) have been developed to *detect* specific markers or to distinguish between normal and cancerous situations. Also, *biosensors based* on *nanoparticles* have been used *in* cancer *detection* to improve conventional analytical procedures. In this chapter, we summarized recent studies relating to development and application of nanoparticle-based biosensors for cancer detection.

Keywords Nanoparticles · Biosensors · Cancer detection · Analytical methods

2.1 Introduction

It is now well understood that the survival of patients with cancer and the effectiveness of treatments strongly depend on the stage of the tumor at the time of diagnosis (The Lancet Respiratory n.d.; Alharbi and Al-sheikh 2014). Therefore, fast, specific, and sensitive cancer diagnosis is pivotally important for clinicians. Accordingly, introducing a biosensor for early detection of cancer has attracted great attention. Biosensor is a system that contains biological recognition elements capable of recognizing target molecule as well as the detector element (transducer) that transforms the signal produced by interaction of the target with the recognition element into quantitative or semiquantitative information (Charbgoo et al. 2016). Nanoparticle-based biosensors named as nanobiosensors could overcome many of the challenges that prevent wide-scale use of biosensors, without any of their major drawbacks. Nanobiosensors have made clinics closer to the ultimate goal of detection that is detecting any cancer biomarker at the level of single molecule or cell. In other word, emerging field of nanotechnology improved particularly transducing element by employing different nanoparticles as signal enhancers. To be specific, nanoparticles (NPs) revolutionized cancer diagnosis due to their unique properties that are different from bulk materials or small ones (Parvanian et al. 2017). The capability of nanoparticles in producing strong signals provided improved cancer imaging systems that have led to accurate image-guided therapies (Baetke et al. 2015). Moreover, nanoparticles are even capable of providing platforms for personalized medicine therapy by enabling preselection and early diagnosis of patients and controlling treatment efficacy (Editorial 2015; Baetke et al. 2015). Applying nanoparticles with unique optical and electrochemical properties and large surface/

volume ratio provided different promising nanobiosensors for timely detection of cancer (Charbgoo et al. 2016). The most common nanoparticles used in biosensing are carbon-based nanomaterials and metallic nanoparticles such as gold and silver nanoparticles (Yang et al. 2018; Wei et al. 2017).

In this chapter, current applications of nanoparticles in fabrication of different effective sensing devices for cancer detection are discussed.

2.2 Nanoparticle-Based Biosensors for Cancer Detection

There are different types of cancer biomarkers that could be detected by nanoparticle-based biosensors such as peptides (Martorella and Robbins 2007; Xu et al. 2017; Yang et al. 2016a), carbohydrate antigen, and genetic biomarkers (Li et al. 2017; Ki et al. 2017; Hu et al. 2015). Considering the properties of these biomarkers in physiological samples, different types of biosensors were designed according to the mechanism of transducing element. Here, we discuss different nanoparticle-based biosensors capable of detecting biomarkers of cancer.

2.2.1 Optical Nanoparticle-Based Biosensors for Cancer Detection

Among different types of biosensors, optical ones are more easily constructed and provide advantages such as low cost, high performance, small size, and high sensitivity and selectivity. Optical nanobiosensors are the most appropriate substituent for traditional analytical techniques. They show the presence of a target molecule in the form of a photo-signal such as color change, absorbance, fluorescence, polarization, and reflective index using nanotechnology.

2.2.2 Colorimetric Nanoparticle-Based Biosensors

Detection of biological components in colorimetric biosensors will be performed through color changes. In this strategy, different nanomaterials like AuNPs, magnetic nanoparticles (MNPs), and quantum dots (QDs) were used. Due to strong light absorption properties, AuNPs are widely used in this type of nanobiosensors (Nekouian et al. 2014). Nekouian et al. detected overexpression of fibronectin (FN) in extracellular matrix (ECM) of lung carcinoma tissue using anti-hFN-Ab conjugated AuNPs. In the presence of high levels of FN in the ECM, anti-hFN-AuNP formed a network in ECM mimicking AuNP aggregation and consequently color change (Nekouian et al. 2014). Another colorimetric method based on distance-dependent color change of AuNP was reported for cancer-related point mutation in

KRAS gene. In this study, poly dT-AuNPs (as probe 1) could hybridize with polyA containing probe 2. Probe 2 is specifically complementary to wild-type KRAS; thus it could capture wild-type target which was previously biotinylated after extracting from the cells. Therefore, in the presence of normal cell, a sandwich of B-KRAS/probe2/probe1-AuNP could be formed. In this study, streptavidin-MNP (S-MNP) was also used to improve color change of AuNPs. The biotinylated KRAS/probe2/probe1-AuNP was attached to the S-MNP, bringing AuNPs close to each other in the magnetic field generating red color change. However, in the presence of mutant, B-KRAS could not be captured by probe2, and subsequently B-KRAS/S-MNP complex could not capture probe1-AuNP; thus color remained unchanged (Valentini et al. 2013). A lateral flow strip biosensor based on AuNPs was also fabricated for the cancer antigen 19-9 (CA 19-9) detection. In the presence of CA 19-9, the first Ab which was labeled with AuNP would be attached to the target followed by conjugation to the second Ab (Ab2) that was immobilized on the test line of the strip. The assembly of AuNPs on the test line produced red color. The control line of the strip was produced by immobilizing Goat anti-mouse IgG that cannot recognize AuNP-Ab1/CA 19-9, so color change would not occur (Baryeh et al. 2017).

2.2.3 Fluorescent Nanoparticle-Based Biosensors

The most common optical nanobiosensors are fluorescent-based ones (Wang et al. 2017a, 2018; Qiu et al. 2017). They detect different types of biological molecules using organic/inorganic fluorophores and also NPs such as SiNPs, AgNPs, AuNPs, and quantum dots (QDs) (Gharatape and Yari 2017; Huertas et al. 2017). Nanoparticles help fluorescent biosensors providing more sensitive and photobleaching-resistant detection. For example, streptavidin (St)-conjugated FITC-labeled SiNPs capable of attaching to biotinylated aptamer (B-aptamer) was used for hepatoma cells detection. SiNP enhances FITC fluorescence signal and dedicates photobleaching resistance (Hu et al. 2017). Using Si nanosubstrate (SiNS), Huertas et al. interestingly designed a fluorescent-based biosensor for detecting mRNA alternative splicing of *Fas* gene (Huertas et al. 2017). This gene introduces two different isoforms as *Fas567* and *Fas57* which are apoptotic and antiapoptotic proteins, respectively. *Fas57* overexpression in cancer cells leads to aggressiveness (Owen-Schaub et al. 1998). Two different ssDNA probes were attached to separate channels of SiNS that has a strong fluorescent signal. The probes were complementary to the special exon junction sequences on each isoform. Attachment of any mRNA isoform provided a particular phase shift demonstrating its presences. This is a very innovative design; however, for real sample detection, a mRNA fragmentation procedure is necessary (Huertas et al. 2017).

Moreover, there are some reports on application of AgNPs in fluorescent-based nanobiosensors. Ag nanoclusters were conjugated to a ssDNA composed of two different fragments for P53 single mismatch mutation. The ssDNA contained one specific DNA sequence (probe) complementary to target p53 and two cytosine-rich

rings at its 3′ and 5′ end. It is demonstrated that cytosine-rich sequences anchor AgNPs and provide fluorescent AgNCs through chemical reduction reactions. In the presence of wild-type p53, a rigid duplex was formed leading to better prevention of AgNC bleaching; thus, a stronger fluorescent signal could be observable. Mutant p53 formed loose duplex causing reduction in fluorescent signal of AgNCs-ssDNA (Hosseini et al. 2017). DNA-AgNCs are widely used in biosensor fabrication since they have tunable emission spectrum, high photostability, and high biocompatibility (Xu and Wei 2017; Yang et al. 2016b). AgNCs play role as label-free reporters overcoming the shortcoming of DNA fragments such as low signal transfer property. AgNC-DNA was also used in fluorescent aptasensors without using PCR or RCA signal amplification strategies for highly sensitive detection of carcinoembryonic antigen (CEA). The target triggers a recycling process to break the pair of AgNCs, leading to decline of the fluorescence intensity of AgNCs. Simultaneously, release of abundant G-quadruplex sequences results in enhancement of the fluorescence intensity of thioflavin T (THT) as THT binding to G-DNA increases its intrinsic fluorescent property. This label-free, enzyme-free aptasensor achieved high sensitivity by using two types of fluorescent probes, THT and AgNCs (Wang et al. 2017a) (Fig. 2.1).

Further, using AgNPs, optical nanofibers were fabricated to immobilize anti-telomerase Ab (Ab1-AgNPs) for detecting telomerase overexpression in breast cancer

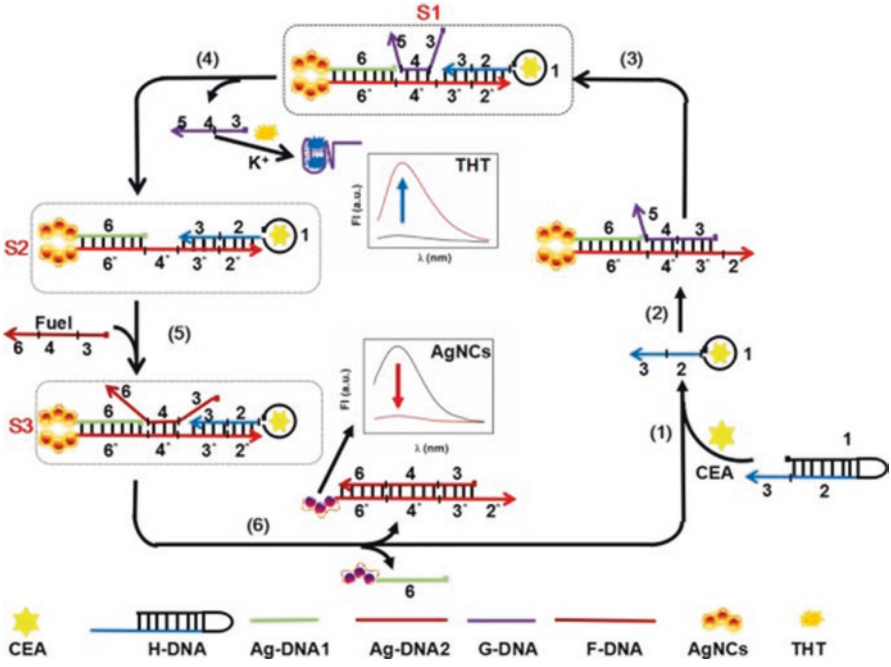

Fig. 2.1 Schematic representation of optical aptasensor based on two label-free probes (THT, AgNCs) using recycling amplification strategy for CEA cancer biomarker detection. (Reprinted with permission from the published work of (Wang et al. 2017a))

single living cell. Ab1-AgNPs were transferred into nucleus of MCF7 cell to bind telomerase followed by conjugation with second Ab which was labeled with alkaline phosphatase through biotin–streptavidin bridging. More telomerase in one MCF7 live cell nucleus provides more fluorescent signal (Fig. 2.2) (Zheng and Li 2010).

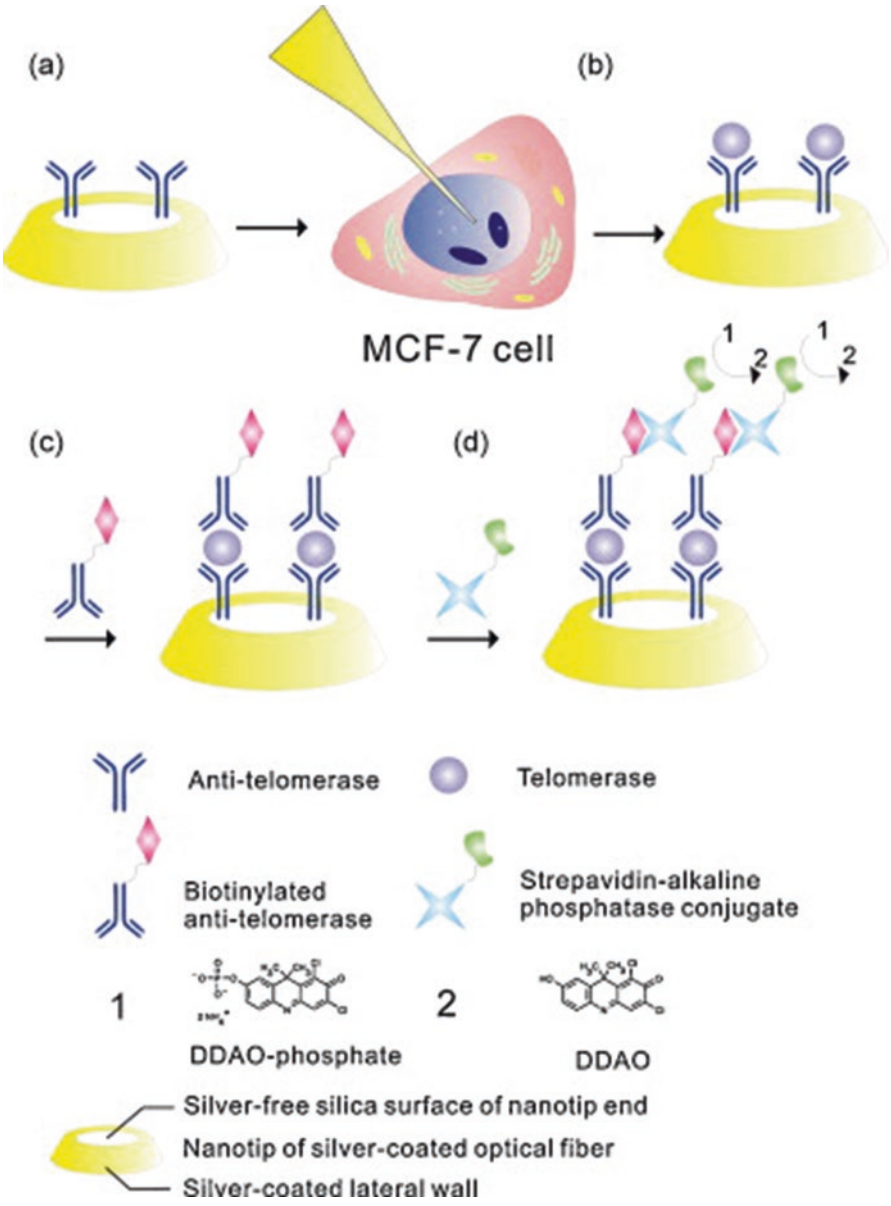

Fig. 2.2 Schematic representation of telomerase detection using Ag nanofiber-based fluorescent biosensors. (Reprinted with permission from (Zheng and Li 2010))

Since AuNPs act as both quencher and carrier, they are also attractive nanoparticles in fluorescent-based nanobiosensors. Using FERT strategy, Ab-conjugated AuNPs and FITC-conjugated Ab were able to detect CEA. When FITC-Ab/CEA/Ab-AuNPs complex was formed, quenching of FITC fluorescence occurred; thus, detection of CEA with LOD of 0.1 ng/ml was accomplished (Wang et al. 2018). AuNPs could also carry fluorescent molecular beacons (MB) in order to detect miRNAs in gastric cancer cells. An AuNP-conjugated ssDNA probe could capture a cye-3-labeled MB resulting in quenching of cye-3 fluorescence by AuNPs. In the presence of target miRNA in the cell cytoplasm, the probe released the MB and captured the target so the fluorescent signal of cye-3-MB could be observable within the cells. It is demonstrated that using star-like AuNPs increased the MB loading level 4.5-fold higher than spherical AuNPs (Ki et al. 2017). Moreover, Fe_3O_4/AuNPs are also used for fluorescent-based early detection of cancer by targeting DNA methylation process in adenomatous polyposis coli (APC) gene as a well-known tumor suppressor. Fe_3O_4/AuNPs conjugated ssDNA probe capable of hybridizing with CpG islands of APC in combination with dipyridamole as fluorescent label were used in this biosensor. Dipyridamole intercalated within duplex of DNA both in methylated and nonmethylated sequences. However, dipyridamole intercalation in methylated duplex decreased the fluorescent signal while its attachment to nonmethylated DNA duplex increased the fluorescent intensity. The reason is that methyl groups as electron donors alter the orientation and interaction of dipyridamole with DNA leading to decrease in the fluorescent emission intensity (Dadmehr et al. 2014).

Another nanoparticle that plays a role in fluorescent-based detection devices is QD. In an interesting design, QDs were used to detect telomerase activity that is correlated with 85% of tumors. In the presence of telomerase, the 3′ end of an immobilized probe was elongated so it could capture a biotinylated probe in the solution. Then, by addition of streptavidin to the duplex, biotinylated QD encapsulated in liposome could attach to the duplex. Following addition of Triton X100, liposome was lysed and then QD released thereby increasing the fluorescent intensity remarkably (Zavari-Nematabad et al. 2017). As a type of QD, carbon dots (CDs) wrapped by AS1411 aptamer could also detect cancer cells overexpressing nucleolin on their surfaces. In the presence of tumor cells, AS1411 aptamers left CDs and captured the cells overexpressing nucleolin on their surfaces leading to detectable fluorescent signal of CD (Motaghi et al. 2017). CDs in combination with AuNPs were also used for CA125 cancer biomarker detection using FRET approach (Hamd-Ghadareh et al. 2017). However, it is demonstrated that QDs provide more sensitive nanobiosensors than CDs particularly for cancer cell detection (Motaghi et al. 2017).

2.2.4 *Surface-Enhanced Raman Spectroscopy (SERS) Nanoparticle-Based Biosensors*

Raman spectroscopy (RS) provides a label-free spectral technique for direct detection of targets. Using RS, signal/noise ratio is very desirable compared to fluorescence and other methods of detection; however, its sensitivity can be improved by about ten orders of magnitude applying surface-enhanced RS (SERS) (Cottat et al. 2015). In SERS, a unique property of metal-based nanoparticles is exploited in which their specific interactions with light and consequent excitation of surface plasmon results in high electromagnetic field at the NPs surface. This electromagnetic field enhances Raman scattering of any molecule at the NPs vicinity, enabling single molecule detection of cancer (Lin et al. 2014). SERS provides several advantages such as narrow spectral peaks, detection of multiple labels using one source, and photostability of labels without bleaching (Lin et al. 2014). Moreover, SERS can provide even label-free detection systems.

Among different metal NP, AuNPs are widely used as SERS active structures. For example, antimanganese superoxide dismutase (MnSOD) aptamer-coated gold nanostructures, which were synthesized by electron-beam lithography, was reported to distinguish MnSOD in serum and saliva with LOD of 10 nM based on SERS (Cottat et al. 2015).

From different scattering modes of SERS including nonpolarized and polarized, it is demonstrated that polarized SERS is more sensitive for distinguishing cancer cells from normal ones with accuracy of 91.6% in blood serum (Lin et al. 2016). SERS-based nanobiosensors are also capable of analyzing stage of tumor which then can be applied for early-stage distinguishing between cancer and noncancerous cells (Lin et al. 2014). In nasopharyngeal cancer (NPC), blood SERs predicted T1 stage vs normal cells with accuracy, sensitivity, and specificity of 83–84% using AuNPs and principal component analysis combined with linear discriminant analysis (PCA-LDA) method in three groups including healthy individuals, early-stage (T1) patients, and advanced-stage (T2-T4) patients. Different cells provided different pattern of Raman intensity signal (Lin et al. 2014).

AuNPs were also combined with graphene in SERS-based detection of cancer. Manikandan et al. introduced an Au nanohexagon-doped graphene sheets capable of distinguishing between normal, cancer, and cancer stem cells based on enhancing Raman signal. Graphene is a strong Raman enhancer due to good performance in preventing fluorescent noise in resonance Raman. It also has easy and low-cost preparation. The cell interacted with this SERS substrate through hydrogen bonding and electrostatic interactions causing alteration in intensity of peaks in SERS curves. This nanobiosensor provided 5.4-fold increase in the detection limit of breast cancer cells (BCCs) and 4.8-fold increment in sensitivity for breast cancer stem cells (BCSCs) detection (Manikandan et al. 2014).

Furthermore, there is a report on use of silver nanoparticles (AgNP) for SERS-based detection of cancer. Wang et al. used a Raman-labeled ssDNA hairpin as a probe that was attached to AgNPs trough SH group. The configuration resulted in

strong SERS signal since Raman label was in close proximity of AgNPs surface. In the presence of target *erbB-2* and *ki-67* as genetic breast cancer biomarkers, hairpin was broken, and hybridization of *erbB-2* and *ki-67* with probe occurred resulting in SERS signal quenching. However, this report provided LOD of 3 mM which is not remarkably sensitive (Wang and Vo-Dinh 2009).

2.2.5 Surface Plasmon Resonance (SPR) Nanoparticle-Based Biosensors

As one another special property of metallic NPs, SPR is widely used for designing highly sensitive nanobiosensors. SPR is produced when incident light interacts with electrons on the metal NPs surface in their conducting band (Hong et al. 2013). The resonance confines a magnetic field that is very sensitive to alterations of dielectric microenvironment. Optical spectroscopy is an appropriate method for detecting the changes in the magnetic field resulting from interactions of molecules with the surface of metallic NPs (Hong et al. 2013). Among metal NPs, AuNPs are widely used for providing SPR-based biosensors carrying different recognition elements such as Ab, peptides, or aptamers.

As a peptide recognition element, tumor-associated antigen (TAA) was attached to Au nanodisks in order to detect autoantibodies immediately secreted by the immune cells after tumor appearance in colorectal cancer (CRC) using SPR-based nanosensors. This system could provide real-time detection of CRC biomarkers with LOD of 1 nM (Soler et al. 2016). In another design, a peptide which could be cleaved by membrane type 1 matrix metalloproteinase (MT1-MMP) was conjugated to AuNP substrate in order to detect invasive cancer cells. MT1-MMP anchors on the invasive cells and is an appropriate biomarker of cancer. In the presence of cancer cell lysate, MT1-MMP cleaved the peptide attached on AuNP substrate producing a blue shift in optical spectrum. As MT1-MMP and cancer cells concentration increased, λmax shift of SPR spectra also increased which resulted in highly sensitive detection of MT1-MMP with LOD of 1 nM (Hong et al. 2013).

Ab-attached AuNPs were also reported to provide surface plasmon-based nanobiosensors. AuNP-deposited nanoporous aluminum oxide chip was used to immobilize an Ab against serum amyloid A1 for lung cancer detection based on SPR. Attachment of target to Ab changes the reflective index, which brought about a shift in resonance spectrum. The LOD of nanobiosensor was 100 ag/ml showing that the sensor was very sensitive for lung cancer detection based on SPR (Lee et al. 2015). Zhang et al. further synthesized a gold nanohole with immobilized anti-PSA antibody as Ab1 for PSA detection in sandwich-like approach. The second antibody (Ab2) was fluorescently labeled so this nanobiosensor was a surface plasmon-enhanced fluorescence (SPFS). Synthesizing nanohole substrate with AuNPs resulted in condensing of electromagnetic field in the Au nanohole that finally provides detection limit as low as 140 fM (Zhang et al. 2017).

Moreover, aptamer conjugated AuNPs have also attracted attention for SPRS-based detection of miRNAs in cancer. For example, an individual AuNP was attached to single-strand oligonucleotide through S–Au bond. The probe, which was against miRNA-21, could form hairpin structure. In the presence of target, hairpin was broken, and miRNA hybridized with the probe generating a red shift in SPR signal (Hu et al. 2015). AuNP in composition with graphene oxide (AuNP-GO) was also reported to immobilize ssDNA molecules for miRNA-141 and small molecule adenosine detection in sandwich-like approach. ssDNA1-attached AuNP-deposited GO was immobilized onto an Au film substrate. In the presence of target, probe 1 captured the target miRNA-141 followed by hybridization of ssDNA2 as probe2, with miRNA-141. The probe 2 was also attached to a layer of AuNP-deposited GO. Using two layers of GO-AuNPs improved signal amplification process and provided LOD in picomolar range (Li et al. 2017). This approach introduced a platform capable of detecting small molecules such as adenosine based on SPR which could potentially overcome the problem of SPR-based biosensors. Typical SPR-based biosensors are not appropriate for detecting small molecules because they cannot change the dielectric constant as much as required due to small interactions with surface of NPs (Gharatape and Yari 2017).

In addition to AuNPs, AgNPs are also used in designing SPR-based nanobiosensors. With a simple approach, anti-HE4 Ab molecules were attached to AgNPs for human epididymis secretory protein 4 (HE4) detection which is an ovarian cancer biomarker (Yuan et al. 2012). A sensitive detection was obtained with LOD of 4 pM with a rapid, low-cost, label-free, and portable screening method (Yuan et al. 2012). However, AgNPs are not as strong as AuNPs in amplification of signal for SPR-based signals.

2.2.6 Electrochemical Nanoparticle-Based Biosensors

Electrochemical biosensors offer high performance, ease of preparation, rapid feedback, and possibilities for miniaturization thus have attracted great attention among researchers (Ibau et al. 2017). These types of biosensors provide label-free detection that is a preferential sensing approach because the signal intensity of labeled biosensors is affected by different parameters such as temperature, pH, and incubation time that limited their uses in various environmental conditions (Charbgoo et al. 2016).

2.2.7 Protein Biosensing

Prostate-Specific Antigen

Prostate-specific antigen (PSA) or human kallikrein 3 (hK3 or KLK3) is the common important serum biomarker for prognosis, screening, and monitoring posttreatment of prostate cancer. PSA is a 33-kDa androgen-regulated serine protease of the

kallikrein family that is produced in the ducts and acinar cells of the prostate. Wang et al. in 1979 reported that PSA can be used as prostate biomarker (Wang et al. 1979). The precursor employed for prostate cancer detection is to monitor the PSA concentration in the human serum. A healthy prostate normally leaks only minute amount into the circulatory system, typically lower than 4 ng/ml; thus, 4 ng/ml is the internationally recognized threshold value for prostate cancer occurrence. In 2011, first application of nanotechnology in PSA electrochemical detection was reported using Au nanoelectrodes. One approach for improving electrochemical detection of PSA using nanoparticles is to modify electrodes with different nanoparticles like AuNPs to produce nanoelectrodes. Nanoelectrodes provide high surface area to communicate with electroactive redox molecules, enhance electron transfer from these molecules to the electrode resulting in high sensitive detection of target molecules. Smaller electrodes transfer higher mass and current density because of thinner depletion thickness. When a biomolecular catalytic reaction is in progress, the high mass transfer is important since it ensures that catalytic activity is not controlled by substrate diffusion onto electrode surface. Nanoelectrodes enhance the diffusion of substrates to the electrode by radial diffusion, thus providing accurate information about the electrochemical response of enzymatic activities (Triroj et al. 2011). Accordingly, using AuNP-deposited alumina electrodes, low levels of PSA (270 fM) in microfluidic environment could be detected in less than 7 sec with dynamic range of 0–50 nM (Triroj et al. 2011). Low-cost carbon ink electrodes were also deposited with AuNPs for more sensitive detection of PSA with LOD of 0.5 fg/mL. AuNPs deposition on carbon ink electrode provided high active surface for anti-PSA molecule immobilization in addition to increasing the electrode conductance (Truong et al. 2012). Other nanoparticles such as iron oxide nanoparticles (IONP) were reported to improve and modified ITO electrodes providing fg/ml detection of PSA (Blel et al. 2016). Nowadays, hybrid nanocomposites are used for improving electrodes functionality. A composite of self-assembled peptide nanotube (PNT), AuNP, and polyaniline (PANI) (PANI/AuNP-PNT) was used to modify a pencil graphite electrode (PGE) for PSA detection with LOD of 680 pg/ml. PSA concentration in PBS was measured with electrocatalytic activity of H_2O_2 catalyzed by HRP on anti-PSA attached to PSA in sandwich similar to the second anti-PSA on PANI/AuNP-PNT-PGE. Both PNT and AuNP have unique electronic properties promoting electrochemical activity of PGE (Vural et al. 2017).

Another approach for improving electrochemical detection of PSA using nanoparticles is to use them as signal enhancers. AuNPs are widely used as signal amplifier in electrochemical biosensors. In an interesting "signal on" design, PSA was detected with LOD of 60 fg/ml using AuNPs as signal amplifiers. First, a peptide that is cleavable with PSA as protease was immobilized on GCE. Then, AuNPs were attached to the peptides followed by the addition of positively charged surfactant, CTAB, to cap AuNPs with positive charge which could then adsorbed the negatively charged $[Fe(CN)6]^{3-/4-}$. This coverage results in lower resistance in electron transfer and impedance. In the presence of PSA, peptide is cleaved releasing AuNPs which then results in higher electron transfer resistance. Signal amplification strategy induced by AuNPs exhibited high sensitivity and reliability (Wang et al. 2015).

Interestingly, AuNPs are used in dual amplified strategies for sandwich-type PSA detection. In this approach, an anti-PSA aptamer was immobilized on gold electrodes in order to capture PSA target. 4-Mercaptophenylboronic acid-coated AuNPs (MBA-AuNP) capable of forming covalent bonds with diols of captured PSA as a glycoprotein were used as the first signal amplifier. The second signal enhancer was electroactive dopamine-coated AuNPs (DA-AuNPs) capable of forming bonds with boronic acid moiety of MBA-AuNP. This method is very sensitive since many DA-AuNP molecules could be attached to MBA-AuNPs considering that each DA-AuNPs carries large number of electroactive dopamine (Xia et al. 2013).

Recently, combination of these two strategies (using nanoparticles as electrode surface modifiers and as signal enhancers/quenchers) is extensively reported for PSA electrochemical detection. Applying AuNPs as signal enhancers and MWCNT-modified GCE for sandwich immunosensing of PSA provided LOD of 5.7 pg/ml. PSA was detected by first antibody-MWCNTs, and then second antibody (Ab2), conjugated to AuNPs attached to the immobilized PSA on the MWCNT-GCE, and formed AuNP-Ab2-PSA-Ab1-MWCNT-GCE sandwich. This composite increased the current which was proportional to PSA concentration (Yang et al. 2015). MWCNTs improved the electron transfer on the GCE (Yang et al. 2015; Nur Topkaya and Ozkan-Ariksoysal 2016), while AuNPs act as carriers for immobilizing high levels of Ab2 (Yang et al. 2015). Moreover, photoelectrochemical immunosensing of PSA using CdSe-sensitized TiO_2-NPs as photoelectrochemical matrix and dopamine–melanin-attached AuNPs as signal quenchers was reported recently (Dong et al. 2017). This design provides dual signal quenching: first by strong energy transfer between AuNPs and CdSe QD and second by inducing steric hindrance between ascorbic acid as electron donor and electrode surface that resulted from attachment of dopamine–melanin–AuNPs. The biosensor provided LOD of 2.7 pg/ml for PSA detection (Dong et al. 2017). In another study, WO_3-AuNP was used to modify ITO and immobilize Ab1. Reduced GO-QDs were also used for signal amplification and immobilizing Ab2. This design increased the photocurrent and introduced LOD of 2.6 pg/ml (Wang et al. 2017b).

CeO_2 mesoporous nanoparticles (CeO_2mNPs) are used as electron donor encapsulating toluidine blue (TB) as the electron transfer mediator while immobilizing Ab1. In order to prevent TB leakage, ionic liquid-doped carboxymethyl chitosan (CMC/ILs) was added to CeO_2mNPs which produced TB/M-CeO_2/CMC/ILs complex. Furthermore, AuNP-QD-graphene-modified GCE was used for Ab2 immobilization by sandwich immunoassay for sensitive PSA detection with LOD of 0.16 pg/ml (Wei et al. 2017). The most sensitive electrochemical sensor for PSA detection was reported by using AuNP-functionalized nitrogen-doped graphene quantum dots (Au@N-GQDs) as platform for immobilizing Ab1 on GCE and Au@Ag-Cu_2O as Ab2 immobilizers, signal amplifiers, and enhancer of electrocatalytic activity toward the reduction of hydrogen peroxide (H_2O_2) for the amplified detection of PSA. The design provided ultrasensitive method of PSA electrochemical detection with LOD of 3 fg/ml (Yang et al. 2018).

It should be noticed that the nanomaterial selection for nanobiosensor design is pivotally important otherwise it negatively affects the LOD of PSA detection (Tezerjani et al. 2016).

Recently, some biosensors for early diagnosis of prostate cancer by simultaneous detection of PSA with other biomarkers such as VEGF are also introduced (Pan et al. 2017).

Cancer Antigen 15-3

Unique properties of nanoparticles such as AuNPs is further used for cancer antigen 15-3 (CA 15-3) detection in breast cancer samples. CA 15-3 is the sheddable form of MUC1 that plays role in cell protection and lubrication. However, its overexpression in cancer cells introduced it as a cancer diagnosis biomarker. Using AuNPs in electrochemical sensing of CA 15-3 provided high surface area; thus, large number of recognition element could be immobilized. Additionally, AuNPs due to higher electrical conductivity, producing more detectable signal play the role of signal amplifier (Selwyna et al. 2013). With the same LOD (5 u/ml), sandwich-type detection of CA 15-3 was also reported in dual target diagnosis (Wu et al. 2014; Cui et al. 2014; Ge et al. 2012). For example, applying AuNP-doped carbon electrode, both CA 15-3 and Her2 could be detected by monoclonal anti-CA and HER1 as Ab1 and alkaline phosphatase-labeled antibody as Ab2 (AP-Ab). AP converts Ag^+ to Ag, and the resulted signal was deposited on the electrode surface at the site of the enzyme thereby avoiding the cross talk between electrodes (Marques et al. 2018).

Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein which has specific cancer-associated carbohydrate alterations when compared with its normal counterpart. Using lectin or monoclonal antibodies, carbohydrate change in glycoproteins can be detected for pathological study of carcinogenesis. This glycoprotein is detected in 60–70% of tumor cells including ovarian carcinoma, breast tumors, cervical carcinomas, and colon tumors. A sandwich-type electrochemical immunosensor with the anti-CEA Ab1 as capture antibody which was attached to magnetic NPs (MNPs) was used for CEA detection. The detection Ab2 was labeled with HRP and further conjugated to AuNPs as signal enhancers. The sensor detected the electron transfer current produced by hydrolysis of H_2O_2 that resulted from attachment of AuNP-Ab2-HRP to CEA providing LOD of 5 ng/mL (Jin et al. 2014). Lectin usage as a CEA recognition element instead of antibodies is preferred due to limitations of Ab. Compared with Ab-based immunosensors, the lectin-based nanobiosensors could detect both the content and the abnormal glycosylation of the tumor markers. One sandwich-type design for CEA detection was introduced, while the recognition element was lectin attached to AuNPs–carbon electrodes (Zhao et al. 2016).

Other Protein/Peptide Biomarkers

Some other peptide biomarkers are also introduced as the candidates of cancer diagnosis using NPs. For example, it is demonstrated that estrogen receptor alpha (ERα) has an important role in initiation and progression of cancer. Therefore, AuNP-modified carbon electrodes were coated with anti-ERα aptamer for producing an electrochemical biosensor for breast cancer detection. After capturing of ERα, the HRP-labeled anti-ERα Ab-coated magnetic NPs were added, and finally the detection with LOD of 10 fg/ml was reported (Uliana et al. 2018). Furthermore, interleukin 8 as a biomarker of salivary oral cancer was also detected using AuNP-doped reduced graphene oxide (AuNP-rGO) in an electrochemical immunosensor. AuNP-rGo nanocomposite provides fast response and high sensitivity because of electron transfer improvement, high surface area, and presenting functional groups for immobilizing recognition elements (Verma et al. 2017).

2.2.8 Genetic Biomarkers Biosensing

As genetic biomarkers of cancers, both RNA and DNA molecules were selected as targets for cancer detection. Specifically, miRNAs are now well-known biomarkers of different cancers. They are released from specific types of cancers, and their detection leads to diagnosis of cancer (Paul et al. 2018). Approximately, 1200 different miRNA molecules are identified, from which miRNA-21, miRNA-141, and miRNA-155 are strongly related to different types of cancer (Ghanghoria et al. 2018). Accordingly, labeled/label-free and enzymatic/enzyme-free approaches are introduced for their detection. As a simple design, an immobilized DNA probe on poly(JUG-co-JUGA)-MWNT-modified GC electrode was used for the detection of miRNA-141 with LOD of 8 fM. Poly(JUG-co-JUGA) provided high surface area, and MWNT improved electron transfer capacity of the electrode (Tran et al. 2013). Moreover, multiple signal amplification approaches for miRNA detection were also introduced. Combining polymerase extension method with alkaline phosphatase and AuNPs, an excellent nanobiosensor was reported in which miRNA was hybridized with the immobilized capture probe and worked as a primer for template extension by polymerase and dNTPs. Since some dNTPs were biotinylated, alkaline phosphatase could attach to the double strand of miRNA/DNA through streptavidin-AuNP as a linker. This method provided very high specificity by detecting even single nucleotide difference, however, showed LOD of 99.2 fM (Peng et al. 2014) that is not as low as expected. This may be attributed to the limitations of enzymatic approaches. However, there are some enzymatic approaches that reported OD as low as 0.14 fM (Hu et al. 2016). In another design, an immobilized probe on Au electrode surface captured the first part of target miRNA-155, while the second probe which was aminated was hybridized with the other part of the miRNA-155. Then, carboxyl groups of grapheme quantum dots (GQD) conjugated electrostatically with NH_2-DNA probe, providing platform for HRP noncovalent attachment that catalyzed TMB oxidation. This enzymatic reaction resulted

in color change and increased the intensity of the electrochemical signal (Hu et al. 2016). miRNA-21 was also detected by electrochemical nanobiosensors. AuNS-coated GCE that could immobilize a probe against miRNA-21 was synthesized. After target capturing and production of duplex (DNA probe/miRNA), toluidine blue (TB) was intercalated into it as signal amplifier indicator and increased the resistance of electron transfer from $Fe_3(CN)_6$ (Tian et al. 2018). Furthermore, miRNA-21 could also be detected in blood serum of patients with gastric cancer. Li et al. designed three DNA molecules as DNA1, DNA2, and, the third one, DNA3 which was labeled with thiol group at 3′ end and ferrocene at 5′ end. DNA1 could be hybridized with both miRNA-21 and DNA2 as can be seen in Fig. 2.2. In addition to these sequences, the solution contained T4 ligase, T7 exonuclease, and AuNP-coated Au electrode. T4 ligase sealed the nick between iRNA-21 and DNA2 providing a perfect duplex for T7 exonuclease. T7 Exo as a sequence-independent nuclease is able to hydrolyze mononucleotides from the blunt but nor overhang of 5′ end of duplexes. Thus, it cannot work on single-strand nucleotides, and thus, in the sensor, T7 Exo hydrolyzed DNA1 fromDNA1/DNA2-miRNA duplex. DNA1 could be also hybridized with the labeled DNA3, providing 4-nucleotide overhang from the 5′ end of DNA1 that protects it from hydrolysis by T7 Exo. However, the enzyme starts degradation of DNA3 from its 5′ end of DNA1/DNA3 duplex. In this situation, the degraded DNA3 could not attach to AuNPs via thiol groups. In the presence of miRNA-21, DNA1/DNA2-miRNA duplex was formed and DNA1 was hydrolyzed by T7 Exo and no longer hybridized to DNA3; thus, DNA1/DNA3 duplex did not form, and consequently DNA3 was not hydrolyzed by T7 Exo. Therefore, DNA3 could attach to AuNP-coated electrodes and increase the current remarkably (Li et al. 2016) (Fig. 2.3).

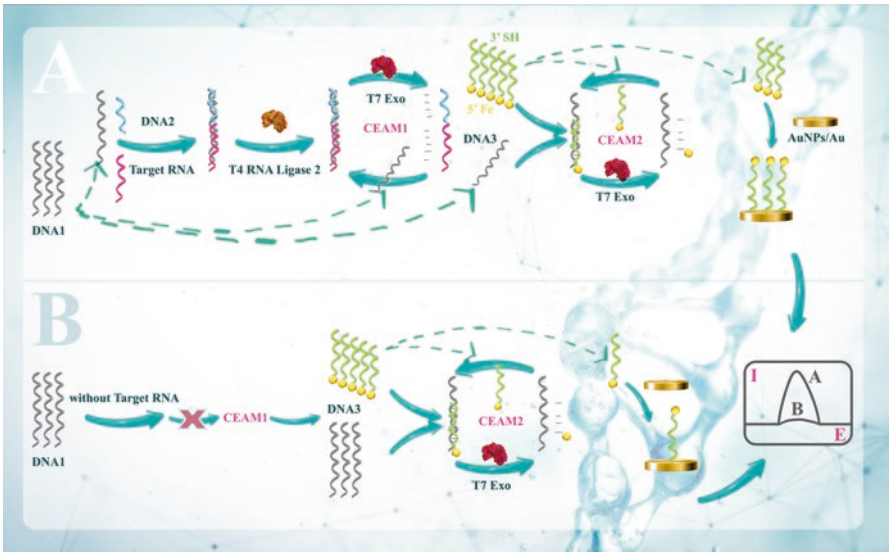

Fig. 2.3 Schematic representation of the DNA probe-based nanobiosensor for miRNA-21 detection. (Redrawn from (Li et al. 2016))

In order to improve accuracy of cancer diagnosis and provide high detection efficiency, multiplex biomarker detection was reported. miRNA-21 and miRNA-141 were simultaneously detected using multifunction MNPs tagged probes coupled with target-triggered hybridization chain reaction (HCR) approach. In this strategy, hairpin probes against the target were immobilized on Au electrode. In the presence of targets, hybridization occurred and hairpins opened, thus providing single strand for binding MNP-complementary sequences (probes). In this approach, HCR could remarkably increase the binding sites of MNP-probe resulting in carrying large number of redox signals followed by amplification of electrical signals (Yuan et al. 2017).

Moreover, nanobiosensors can be used for cancer-associated DNA sequence detection. Saeed et al. developed a sensor for the detection of Her2 overexpressed gene and CD4 as a prognostic marker in breast cancer using AuNP-attached GO-coated GCE. A capture probe 1 was immobilized on AuNPs by thiol binding. In the presence of target DNA, hybridization with probe 1 occurred followed by hybridization of HRP-labeled probe. The attached HRP hydrolyzed the TNB and resulted in increase in resistance of electron transfer, and consequently, the current decreased depending on the DNA target concentration (Saeed et al. 2017). Furthermore, using DNA probe-immobilized QD-modified electrode, chronic myeloid leukemia (CML) was detected. The probe was designed from BCR-ABL oncogene which is overexpressed in CML. The QD generated increased electron transfer kinetics and improved electroactive surface area (Sharma et al. 2013).

2.2.9 Cytosensors

Electrochemical cytosensors have great role in diagnosis of cancer cells due to advantages such as rapid response, high sensitivity, easy operation, nondestructive analysis, and real-time monitoring. Using different nanomaterials provided considerable sensitivity to these sensors.

In this type of sensors, an overexpressed marker or cancer-specific glycons on the cell surface or a cell-secreted molecule can be detected. A lectin-based cytosensor for the detection of living cancer cells was introduced considering sialic acid abundance in tumor cells. Lectin could capture cancer-specific glycosylation of tumor cells in sandwich-type cytosensor, in which AuNPs act as signal amplifier and MWNTs as electrode modifiers (Zhang et al. 2010). MUC1 is also an appropriate candidate for most cancer cells and could capture breast solid tumors. Mouffouk et al. developed an electrochemical cytosensor for breast cancer using self-assembled pH sensitive polymeric micelle loaded with ferrocene as signal generator and amplifier. They conjugated anti-MUC1 Ab on both magnetic NPs and ferrocene-containing micelles. In the presence of MCF7 cells, micelles and MNPs were attached to cells and subsequently were captured under magnetic field. Decreasing the pH of solution triggered the ferrocene release and produced sensitive signal as each micelle contained thousands of ferrocene molecules (Zhang et al. 2018).

As described above, various electrochemical cytosensors have been reported; however, implantable cytosensor capable of real-time monitoring and in vivo

detection has attracted great attention. This approach requires highly biocompatible blocks for the biosensor. For this purpose, peptides are appropriate candidates due to their inherent biocompatibility, biological recognition, and chemical versatility. For example, diphenylalanine (FF), a short dipeptide that can be self-assembled to peptide nanotube (PNT) structure, was used for the detection of K562 cells. FF catalyzes oxidation of H_2O_2 and NADH owing to electroconductivity resulting from its spatially aligned electrons of aromatic ring. PNTs are attached to chitosan (CS) to provide hydrophilic support films, improving morphology and increasing stability. The hydrophilic PNT–CS composite produces an appropriate microenvironment for cell immobilization which is a critical issue for synthesizing an electrochemical cytosensor. K562 cells adhere to PNT on PNT-CS electrode where nanotube enhances the electron transfer from electroactive center of the cells (glutathione) to the electrode due to its conductivity (Lian et al. 2017). In another approach, products that are secreted from live cancer cells can be detected as a criterion of cancer cell presence. For example, since cancer cells produce more H_2O_2 than normal cells, these molecules can be detected in situ real time in live cells as molecular probes of cancer cells. Previously AuNP-decorated nitrogen-doped carbon nanotubes on carbon fiber electrodes was used to detect H_2O_2 secreted from live MCF7 and MDA-MB cells with LOD of 50 nM. Owing to electrocatalytic activity of AuNPs and high electroconductivity of carbon nanotubes, this sensitive detection of H_2O_2 was developed (Fig. 2.4) (Zhang et al. 2018).

2.3 Conclusion

We have discussed nanoparticle-based biosensors for cancer diagnosis. In comparison with conventional analytical methods, NP-based biosensors are simple, flexible, and inexpensive and can be easily fabricated. Implementation of various NPs with interesting properties could provide higher sensitivity, lower detection limit, and wider linear detection range for early cancer diagnosis (Table 2.1).

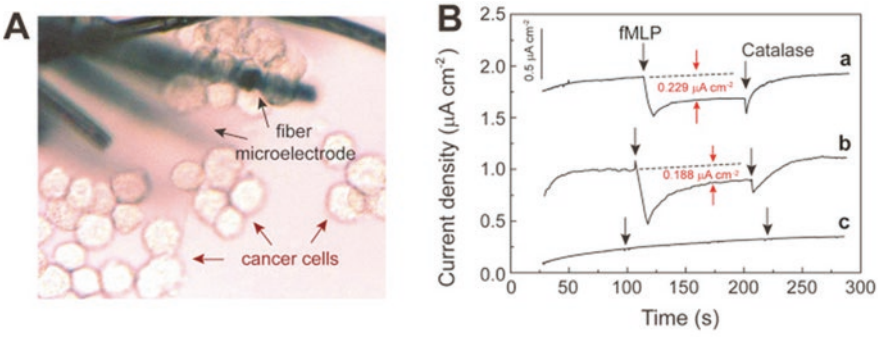

Fig. 2.4 (**a**) Super resolution image of carbon nanofiber electrode in live cancer cell suspension. (**b**) Current change triggered by H_2O_2 secreted from the cells after using two simulators. (Reprinted with permission form (Zhang et al. 2018))

Table 2.1 Different types of nanoparticle-based biosensors for cancer biomarkers detection

	Type	Target	Type of biomarker	Method	LOD	Elements	Ref
Optical nanobiosensors							
1	Optical	Fibronectin of lung carcinoma	Glycoprotein	Colorimetric	–	Color change results from assembly/ aggregation of AuNP	Nekouian et al. (2014)
2	Optical	35G > A point mutation in KRAS gene in lung cancer	DNA (gene)	Colorimetric	20 pM	Color change results in assembly of probe-AuNP	Valentini et al. (2013)
3	Optical	CA 19-9 in pancreatic and colorectal cancers	Protein	Colorimetric	5 u/ml	Color change results in assembly of probe-AuNP	Baryeh et al. (2017)
4	Optical	HepG2	Hepatoma cells	Fluorescent	–	St-FITC-SiNP/B-aptamer/cells	Hu et al. (2017)
5	Optical	*Fas 567* and *Fas57* mRNA isoforms	RNA	Fluorescent	580 fM	mRNA/ssDNA-SiNS	Huertas et al. (2017)
6	Optical	P53 mismatch mutation	DNA	Fluorescent	1.3 nM	P53 mRNA/ssDNA-cytosine rings-AgNC	Hosseini et al. (2017)
7	Optical	CEA	Protein	Fluorescent	100 pg/ml	CEA/aptamer-H-DNA/ AgNCs-DNA/G-DNA/ THT	Wang et al. (2017a)
8	Optical	Telomerase activity in single live MCF7	Protein	Fluorescent	–	Ab1-AgNPs/telomerase/ alkaline phosphatase-Ab2	Zheng and Li (2010)
9	Optical	CEA	Protein	Fluorescent	100 pg/ml	FITC-Ab/CEA/ Ab-AuNPs	Wang et al. (2018)
10	Optical	miRNA in gastric cancer	RNA	Fluorescent	<10 M	ssDNA-AuNP/ cy3-ssDNA(MB)/ miRNA	Ki et al. (2017)

11	Optical	DNA methylation of APC gene	DNA	Fluorescent	Unmethylated DNA: 0.21 aM Methylated DNA: 0.31 aM	Dipyridamole/ssDNA probe- Fe_3O_4/AuNPs	Dadmehr et al. (2014)
12	Optical	Telomerase activity in A549 cells	Protein	Fluorescent	10 cells	CdTe QD-liposome-biotin/st/biotin-probe2/ probe 1-telomerase	Zavari-Nematabad et al. (2017)
13	Optical	4 T1	Cancer cells	Fluorescent	100 cell/ml	CD-aptamer/cancer cell	Motaghi et al. (2017)
14	Optical	CA125	Protein	Fluorescent	0.5 fg/mL	AuNP-dendrimer-Ab/ CA125/ssDNA-CD	Hamd-Ghadareh et al. (2017)
15	Optical	MnSOD	Protein (enzyme)	SERS	10 nM	MnSOD/aptamer-AuNS	Cottat et al. (2015)
16	Optical	Tumor stages in nasopharyngeal cancer		SERS	–	AgNP	Lin et al. (2014)
17	Optical	Breast cancer/cancer stem cells	Cancer cells	SERS	100 µg/l × 10^4 cells	Au nanohexagon-doped graphene sheets	Manikandan et al. (2014)
18	Optical	*erbB-2* and *ki-67* of breast cancer	DNA	SERS	3 mM	ssDNA-AgNP	Wang and Vo-Dinh (2009)
19	Optical	MT1-MMP	Protein (enzyme)	SPR	–	Glass substrate-AuNR-peptide/MT1-MMP	Hong et al. (2013)
20	Optical	TAA	Proteins	SPR	1 nM	Ab/ TAA-PLL-PEG-AuNS	Soler et al. (2016)
21	Optical	Serum amyloid A1 of lung cancer	Protein	SPR	100 ag/ml	Anodic aluminum oxide-AuNP-Ab	Lee et al. (2015)

(continued)

Table 2.1 (continued)

Type		Target	Type of biomarker	Method	LOD	Elements	Ref
22	Optical	PSA	Glycoprotein	SPF	140 fM	Au nanohole-Ab1/PSA/labeled Ab2	Zhang et al. (2017)
23	Optical	miRNA-21	RNA	LSPR	3 nM	ssDNA-ANP-ITO glass substrate	Hu et al. (2015)
24	Optical	miRNA-141 Adenosine	RNA	SPR	100 pM	Au film-GO-AuNP-ssDNA1/miRNA/ssDNA2-AuNPs-GO	Li et al. (2017)
25	Optical	HE4 of ovarian cancer	Protein	SPR	4 pM	Ab- AgNP	Yuan et al. (2012)
Electrochemical nanobiosensors based on type of detected biomarker							
Protein							
26	Electrochemical	PSA	Glycoprotein	Cyclic voltammetry (CV)	10 pg/ml	AuNP-deposited alumina electrode	Triroj et al. (2011)
27	Electrochemical	PSA	Glycoprotein	Impedimetric	0.5 fg/mL	AuNP-deposited carbon electrode	Truong et al. (2012)
28	Electrochemical	PSA	Glycoprotein	Impedimetric and amperometric	10 fg/mL	IONP-doped ITO electrode	Blel et al. (2016)
29	Electrochemical	PSA	Glycoprotein	Amperometric	680 pg/ml	PANI/AuNP-PNT-modified graphite electrode	Vural et al. (2017)
30	Photoelectrochemical	PSA	Glycoprotein	Photocurrent	60 fg/ml	CTAB-capped AuNPs with PSA cleavable peptide-modified graphite electrode	Wang et al. (2015)
31	Electrochemical	PSA	Glycoprotein	CV, PDV	1.6 pg/ml	DA-AuNPs/MBA-AuNPs/PSA/apt-gold electrode	Xia et al. (2013)

32	Electrochemical	PSA	Glycoprotein	Differential pulse voltammetric (DPV)	5.4 pg/ml	AuNP-Ab2/PSA/ Ab1-MWCNT-GCE	Yang et al. (2015)
33	Photoelectrochemical	PSA	Glycoprotein	Impedimetric and CV and photocurrent	2.7 pg/ml	Dopamine-melanin-AuNPs with QD-TiO_2 NP-modified ITO	Dong et al. (2017)
34	Photoelectrochemical	PSA	Glycoprotein	Photocurrent	2.6 pg/ml	WO3-AuNP-Ab2/PSA/ Ab1-QD-rGO- ITO	Wang et al. (2017b)
35	Electrochemical	PSA	Glycoprotein	DPV and amperometric	0.16 pg/ml	TB/M-CeO2/CMC/ ILs-Ab2/PSA/Ab1-AuNP-QD-graphene-modified GCE	Wei et al. (2017)
36	Electrochemical	PSA	Glycoprotein	Impedimetric and CV and amperometric	3 fg/ml	Au@Ag-Cu2O-Ab2/ PSA/ Ab1- Au@N-GQDs-GCE	Yang et al. (2018)
37	Electrochemical	PSA and VEGF	Glycoprotein and protein	DPV	1 ng/ml 50 fg/ml	Apt-GO-PLLA-Au electrode/PSA, VEGF	Pan et al. (2017)
38	Electrochemical	CA 15-3	Protein	CV, EIS	5 u/ml	Saturated calomel electrode (SCE)-AuNP-Ab/CA 15-3	Selwyna et al. (2013)
39	Electrochemical	CA 15-3 & HER2	Protein	Linear sweep voltammetry (LSV)	5 u/ml 2.9 ng/ml	Ab1-AuNP-carbon electrode/CA-15 13 & HER2/Ag, AP-Ab2	Marques et al. (2018)
40	Electrochemical	CEA	Protein	CV and impedimetric	5 ng/ml	Graphene@MNPs-Ab1/ CEA/HRP-Ab2-AuNP	Jin et al. (2014)
41	Electrochemical	CEA	Protein	Chronoamperometric	0.01 ng/mL	CE@AuNPs-lectin/CEA/ HRP-Ab	Zhao et al. (2016)

(continued)

Table 2.1 (continued)

Type		Target	Type of biomarker	Method	LOD	Elements	Ref
42	Electrochemical	ERα	Protein	CV	10f g/ml	HRP-MP-Ab/ERα	Uliana et al. (2018)
43	Electrochemical	IL-8	Protein	CV and DPV	72.73 ± 0.18 pg/mL	AuNP-rGO	Verma et al. (2017)
44	Electrochemical	miRNA-141	RNA	SWV and CV	8 fM	miRNA/ssDNA probe-poly(JUG-co-JUGA)-MWNT- GC	Tran et al. (2013)
45	Electrochemical	miRNA	RNA	DPV and EIS	99.2 fM	miRNA/ssDNA-B-dNTPs/St-AuNP-B-alkaline phosphatase	Peng et al. (2014)
46	Electrochemical	miRNA-155	RNA	CV and EIS	0.14 fM	HRP-GQD-probe 2/miRNA/probe 1-Au electrode	Hu et al. (2016)
47	Electrochemical	miRNA-21	RNA	DPV	78 aM	TB-miRNA/probe-AuNS-GCE	Tian et al. (2018)
48	Electrochemical	miRNA-21	RNA	DPV	0.36 fM	DNA1/DNA2/DNA3/miRNA/AuNP-Au electrode	Li et al. (2016)
49	Electrochemical	miRNA-21 and miRNA-141	RNA	DPV	0.36 fM and 0.28 fM	DNA1/Fe_3O_4 NPs/Thi and DNA2/Fe_3O_4 NPs/Fc	Yuan et al. (2017)
50	Electrochemical	HER2 DNA	DNA	CV & EIS	0.16 nM	HRP-probe2/probe1-AuNP-GO-GCE	Saeed et al. (2017)
		CD4 DNA			0.23 nM		
51	Electrochemical	CML DNA	DNA	CV	1 pM	DNA target/probe-QD-ITO electrode	Sharma et al. (2013)

Whole cancer cells							
52	Electrochemical	Cytosensor (sialic acid detection on MRC-5, A549, H1299)	Monosaccharide	DPV	1 cell/ml	AuNP-thionine-Lectin/ cancer cell/ lectin-MWNT-GCE	Zhang et al. (2010)
53	Electrochemical	Cytosensor (MUC1 on MCF7)	Protein	CV	10 cell/ml in less than 1 min	Ab1-MNP/cancer cell/ Ab2-ferrocene-loaded pH-sensitive micelle	Zhang et al. (2018)
54	Electrochemical	Cytosensor (K562)	Cancer cell		630 cells mL^{-1}	PNT–CS	Lian et al. (2017)
55	Electrochemical	Cytosensor (MCF-7, MBA-MD-231	Cancer cell		50 nm of H_2O_2	AuNP-decorated nitrogen-doped carbon nanotubes on carbon fiber electrodes	Zhang et al. (2018)

All reported advantages for NP-based biosensors confirm the versatility of this platform for the development of diagnostic kit for clinical applications. Different NPs such as gold NPs, graphene oxide and graphene quantum dots, magnetic NPs, silica NPs, QDs, etc. with diverse characteristics can be implemented as fluorescent/optical probe, quencher, transducer, etc. toward the application in cancer diagnosis due to their superior properties in terms of electronic/thermal conductivity and special luminescence and mechanical properties.

Major biosensor systems summarized here comprise electrochemical and optical sensors.

Commonly, NPs with desirable surface modification can facilitate the detection of interaction between specific biomarker ligand and its receptor for definite cancer cells.

Although numerous studies were conducted on the chemistry, intensity level, and concentration of surface modification of NP-based sensors, the optimal conditions for providing low detection limit and high sensitivity against specific biomarker for cancer cells are not well studied.

Furthermore, the in vivo performance of NPs with diverse surface modifications is complicated and still under examination for better understating.

The crucial concerns regarding NP-based sensors for cancer detection have been identified which include NP biocompatibility, diverse biodistribution of NPs, complicated NPs–cell membrane interaction, stability and biocompatibility of NPs following chemical modifications, verification of safety, nontoxicity, and NPs' fate in vivo.

It could be concluded that if the aforementioned obstacles are addressed appropriately for each given application, the NP-based sensors will meaningfully improve the early detection of cancer.

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Chapter 3
Insights into Nanotools for Dental Interventions

Pooja Jain, Fahima Dilnawaz, and Zeenat Iqbal

Contents

Abstract Orodental afflictions although not life-threatening have recently been found to be clinically associated with a large variety of life style related diseases, adding to the disease burden and leading to poor health outcomes. The disease ramifications might lead to poor quality of life and often go unnoticed till exhibits severe symptoms and evidence of disease associations. Despite being a locoregional affliction, they do not remain restricted to the mouth cavity; rather they have negative effects on distant organs too. Henceforth the disease requires a thorough understanding, and the treatment modalities are devised in a manner to cater mild to severe status of orodental afflictions. Successful treatment of orodental afflictions requires the customized therapeutic approaches which can be borrowed from the nanotechnology. The advancement in nanotechnology and its ramification into

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medicinal and allied fields has brought remarkable changes in the field of dentistry. Additionally, the forays into nanomaterial sciences, biotechnology, and maneuvering through dental nanorobotics have further added impetus to contemporary dental practices. Often the dental infections are progressive in nature and need an umbrella approach for treatment to deliver comprehensive oral hygiene and health. Nanotechnology tools with their aesthetic approach have recently emerged as a favored tool for both practitioners and patients. The treatment protocols could include controlled oral analgesia, dentin replacement therapy, as well as cure for dental hypersensitivity. Interesting development of mechanical dentifrobots for tooth repairing via covalently bonded diamondized enamel ensures robust orodontal environment. Succinctly put, nanotools have opened up avenues in the diagnosis, treatment, and prevention of dental diseases. The development of nanotools allows perfect therapeutic approach which has led to nearly seamless oral health, possibly due to the use of nanomaterials, tissue engineering, nanocomposites, and nanorobots. Nanotools have elicited great impact on restorative dentistry, pharmacotherapeutics, and diagnostic techniques and offers better ways toward maintaining perfect oral health. This chapter focuses on a review of various approaches of nanotechnology which has contributed to the advancements in dentistry. It also focuses on advent of other nanotools which will cater to a variety of dental ailments associated with bony as well as soft tissue defects. It is realized that the nanotechnology offers a perfect tool to treat a multitude of orodental infections.

Keywords Bone grafts · Laser therapy · Nanoanesthesia: Nanofibers · Nanocomposites · Nanodentistry · Nanotechnology · Orodental infections · Orthodontics · Periodontics

3.1 Introduction

The global prevalence of orodental infections has risen enormously in the last few decades leading to reported cases of about 3.5 billion, majorly affecting the population of lower social strata (Dye 2017). Although it is rarely associated with fatal disease outcomes, recent clinical findings have established its foray into systemic complications (Bošnjak et al. 2001). These findings are reasons enough to attract the attention of current researchers who are focusing on oral diseases, customizing their treatment modalities, and progressively designing the advanced drug delivery systems.

Orodental afflictions broadly include the various diseases which attack the gingival and subgingival tissue as well as the dental adnexa. The various dental infections and their progressions can be summarized with the help of Fig. 3.1.

Amelioration of such diseases requires customized treatment which has recently borrowed from the discoveries of nanotechnology armamentarium. The treatment premise primarily depends on the disease stage and types of dental affliction and the

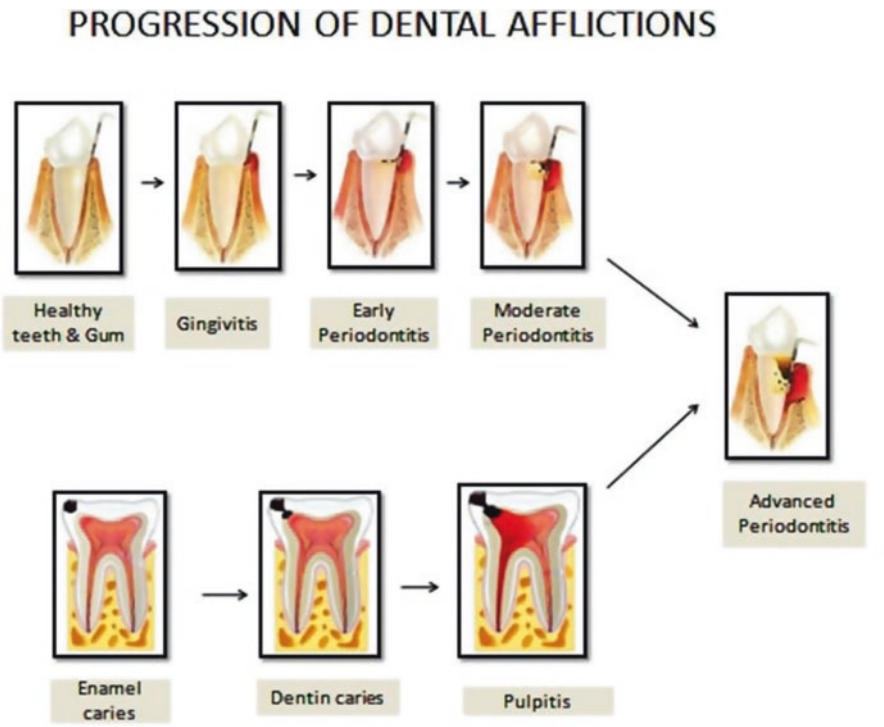

Fig. 3.1 Progression of dental afflictions
The dental afflictions progresses in two ways: In one way, it starts from gingivitis, early periodontitis, then moderate periodontitis, and ultimately severe periodontitis. In another way, it begins with enamel caries, dental caries, then pulpitis, and ultimately advanced stage periodontitis

intervention required. Intrapocket infections like periodontitis with its specific stages and treatment challenges constitute the largest umbrella of diseases in this area. Up till now dental treatments are challenging as people are psychophobic toward dentistry. On account of this fear, they avoid the dental treatment as they are scared for injection and drills that are used in dental clinics. Also it is a fact that conventional methods of root planning, scaling, and plaque removal have limited success and poor patient compliance because of length of procedure and high treatment costs. To ease the discomfort in recent times, dentistry offers period of dynamic changes and growth with the use of ozone in dental treatment. Through ozone therapy, the pain is eradicated significantly. Ozone also stimulates remineralization in case of caries-affected teeth providing potentiality for an atraumatic, biologically based treatment for conditions encountered in dental practice (Gupta and Mansi 2012; Moezizadeh 2013).

The integration of nanotechnology in "dental interventions" ever since its existence has immensely contributed to the advancements in diagnosis, prevention, and treatment of various dental ailments (Uysal et al. 2009). The potential applications of nanotools include an umbrella of dental techniques focused at dentition renaturalization, local anesthesia, orthodontic realignments, permanent hypersensitivity cure, covalently bonded diamondized enamel, oral health maintenance with the help of mechanical dentifrobots, and artificial bone and teeth creation (Rybachuk et al. 2009). There is an increase in opinion that if nanotools are applied to dentistry, it will bring significant advances in the diagnosis, prevention, and treatment of dental disease in numerous ways.

3.1.1 Dental Tissues or Tooth Anatomy

The human teeth are composed of four types of tissues, namely, enamel, dentin, cementum, and pulp. Out of the four, pulp is the only soft, noncalcified connective tissue and contains nerves and blood vessels. And the other three are the hard tissues (Berkovitz et al. 1978).

Enamel is the hard substance or the calcified tissue and covers the dentin in the crown. It is the hardest substance/tissue of human body and composed of 96% inorganic minerals, 1% organic materials, and 3% water. Calcium and phosphorus (as hydroxyapatite) are the two main inorganic constituents. It is devoid of living cells and blood supply and thereby lacks the potential of autoregeneration (Türp and Alt 1998).

Dentin is present in between the enamel and cementum. Dentin is porous in nature and constitutes to the largest portion of tooth. Dentin also consists of 70% inorganic matter and 30% organic matter and water. It is a living tissue and is perforated with microscopic tubules that run between the cement–enamel junction and the pulp. If the enamel gets degraded from the surface, then these tubules start stimulating the sensations of heat and cold thereby generating the sensitivity (Black 1897).

Cementum is the hard bone-like connective tissues, covers the root of tooth, and provides the attachment to periodontal ligament. It is composed of approximately 55% organic material and 45% inorganic material. The major components of inorganic material are the calcium salts. The area where the cementum joins the enamel is called as the cement–enamel junction, and such areas are very sensitive to the external stimuli such as heat and cold (Türp and Alt 1998).

Dental pulp is the soft tissue that develops from the connective tissues of dental papilla and contains nerves and blood vessels. Beneath the crown, the chamber containing pulp is called the pulp chamber and the pulp is called as coronal pulp, whereas inside the root, the pulp is called as radicular pulp. The main function of the pulp is the formation and nourishment of the dentin. It also provides sensation to the

tooth and shows irritation response in terms of inflammation. The nerves and blood vessels pass through the end of radicular pulp to reach the interior of the tooth (Black 1897).

3.1.2 Types of Nanotechnological Approaches

The main approaches which could help in tangible use of nanotools in practice are as follows:

(i) Bottom-up approach; where it involves arrangement of smaller components into complex structures. It focuses on the use of micron-sized nanorobots which could be manipulated for a variety of hands on jobs like instilling local anesthesia, polishing the enamel surface with diamondized covalent bonding, curing of teeth hypersensitivity, dental durability and cosmetic appearance, and use of photosensitizers like quantum dots for hitting the specific targets (Kumar and Vijayalakshmi 2006).
(ii) Top-down approach: where it embraces development of nanostructures of smaller dimensions using larger parts. It primarily involves the use of nanosolutions, nanoneedles, nanofillers, nanocomposites, and bone replacement materials (Sasalawad et al. 2014).

Adper™ single bond plus is the example of dentin adhesive nanosolution with single step application procedure (Perdigao 2007). Suture needles such as Sandvik Bioline RK 91™ needles are developed to a carry nanosized stainless steel crystals to minimize the tissue damage (Suresh et al. 2014). The top-down approach of nanotechnology has application in salivary diagnostics as well, where the saliva can be analyzed by the biochemical sensors. Oral fluid nanosensor test (OFNASET) is the one such automated and integrated system to detect the targeted protein and nucleic acid in saliva (Terry 2004; Wong 2006).

Although it is a futuristic and promising tool in dental practice, patient acceptability and desired success is still elusive to nanotools in dentistry. However, it is marred with a set of unique tasks which encompass engineering, biological, as well as ethical and social issues. The manufacturing and engineering challenge lies in the lack of mass production, precise positioning and assembly of molecular scaling part, manipulating, and coordinating activities of large numbers of independent microscale robots. Simultaneously biological challenges are there for the development of biofriendly nanomaterial that would ensure compatibility with all intricacies of the human body. Although nanotechnology would possess tremendous potential, the social issues of ethics, public acceptance, regulation, and human safety needs to be addressed as it carries a significant potential for misuse and abuse on such a scale that has never seen before. In this review, current status of nanotools in dentistry has been discussed that provides an insight into its future along with the ethical and safety concerns related to it.

3.2 Applications of Nanotechnology in Dentistry

As the nanotechnology includes bottom-up approach and top-down approach, depending upon these approaches, various applications of nanotechnology are explained in the individual sections and summarized in Fig. 3.2.

3.2.1 Applications of Nanotechnology in Restorative Dentistry

Emergence of nanodentistry and its various uses aid in the maintenance of affected oral health through the use of various nanomaterials, tissue engineering, nanotools, and nanorobotics that could lead to achieve a near to perfect oral health. In the following sections, using different nanodevices pertaining to the oral health has been discussed.

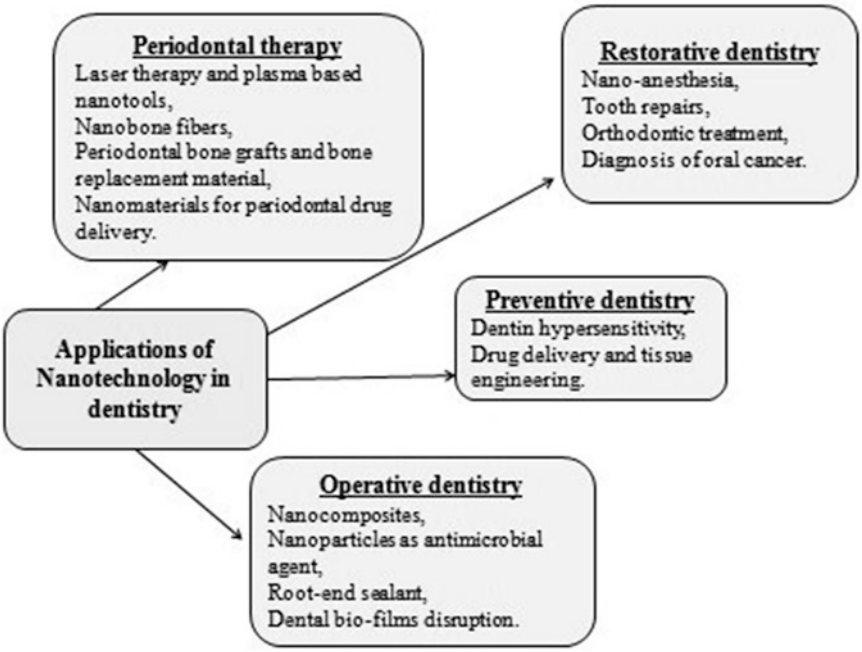

Fig. 3.2 Various applications of nanotechnology in dentistry
Various application of nanotechnology are categorized into restorative dentistry, operative dentistry, preventive dentistry, and periodontal therapy

Nanoanesthesia for Surgical Intervention

The major deterrent for a dental patient is the assumption of the ensuing pain which he/she will face on a visit to the dentist, and therefore in dental practice, the most common procedure is the administration of oral anesthesia. The colloidal suspension of oral nanoanesthesia is comprised of millions of active analgesic nanorobotic particles and administered to the patient's gingiva. After that it comes in contact with the crown or mucosa and the ambulating nanorobots reach the dentin by migrating painlessly through the thick layer of loose tissue at the cement–dentinal junction. These nanorobots then enter the dentinal tubules holes (~1 to 4 microns in diameter) and progress toward the pulp guided by a combination of chemical gradients, temperature difference, as well as positional navigation under the control of the onboard nanocomputer as directed by the dentist. The branching pattern of tubules sometimes gives a significant challenge to navigation. The robotic movements are successful due to the constant motion of natural cells around and inside the teeth. Once it is successfully installed and it has established control over the nerve impulse traffic, the analgesic dental nanorobots can be commanded by the dentist to shutdown the sensitivity for any particular tooth that needs treatment. After the hand held controller, the targeted tooth become numb and treatment procedure is followed. The nanorobot is aspired out after completion of the oral procedure, and the dentist restores all the sensation (Schleyer 2000).

These nanorobotic analgesics provide great patient comfort, reduced anxiety, specific selectivity, as well as controllability to avoid most side effects and complications. Also, it is envisioned that nanorobots will assist dentists in precisely operating the complex cases of microscopic level. And in the future, it will be a prime tool for dentists to practice conventional as well as four-handed dentistry.

Tooth Repairs

Most common dental problem is dental caries, which occurs in all age groups and adversely affects an individual's daily life seriously. The dental caries are slowly progressive and infectious disease that results in loss of dental hard tissue. In conventional treatment the caries are mechanically excavated and then filled up with resins or metals comprising inorganic-base cements as glues. Ideal filling materials are usually similar to the structure and composition of natural tooth (John 2007). The dental hard tissue is composed of inorganic hydroxyapatite (HA) crystal and organic matrix; by the process of biomineralization the hydroxyapatite crystal is orderly oriented. In routine clinical treatments, microleakage occurs with the secondary caries due to the discrepancy in physicochemical property between filled materials and tooth.

Various synthetic materials are available to restore the dental caries. Hydroxyapatite crystal, minerals like amorphous calcium phosphate (ACP), casein protein (CPP) compounds, nanobioactive glass; and the stimulated body fluid could reverse the early caries lesion in dentin or enamel surface (Yamagishi et al. 2005;

Andersson et al. 2007; Vollenweider et al. 2007). Guided tissue remineralization can also be imposed for partially demineralized human dentin where dentin collagen could guide the phase transition of nano-ACP into HA (Tay and Pashley 2008).

With the help of nanotechnology, molecular biomimetics materials were fabricated. These biomimetics materials are based on molecular recognition between genetically engineered peptides for inorganics (GEPIs) and inorganic crystal. Hybrid of GEPI and HA crystal can be used in the assembly of functional nanostructures using their recognition properties to repair dental hard tissue in the future (Zhou et al. 2008).

With the availability of tooth repair materials, dental caries is not a difficult problem to handle, and with the help of nanomaterials, partial to complete restoration is possible and this serves as boon for dental cosmetics industry.

Orthodontic Treatment

In orthodontic treatment sliding a tooth along the arch wire involves more frictional force for its movement. Applying excessive orthodontic force might cause loss of anchorage and root resorption. Redlich et al. (2008) reported the reduction of friction by coating inorganic fullerene like tungsten disulfide nanoparticles (IF-WS2) nanosolution. IF-WS2 is known for their excellent dry lubrication properties. The NP-coated wires offer better friction reduction opportunity up to 54% during the tooth movement. Polymer nanocomposites are used as orthodontic adhesives for increasing mechanical properties, tensile, comprehensive strength, and resistance to fracture. These nanocomposites illustrate excellent optical properties, superior polish ability, and easy handling. These nanofiller nanocomposites can decrease the surface roughness of the orthodontic adhesives. As, the oral cavity is an open growth system, where attachment and colonization of bacteria takes place and it gradually shifts towards disease. The rough surface promotes plaque formation and maturation and strong binding. Therefore decrease in roughness by nanocomposites can dramatically enable very less bacterial accumulation (Quirynen and Bollen 1995; Lee et al. 2004). Nanoinomers are based on resin-modified monomers of acrylic and itaconic acid copolymers which polymerizes via free radical addition and curing by light activation (Korkmaz et al. 2010). The nanocomposites, i.e., Filtek Supreme Plus Universal, and nanoionomers like Ketac ™ N100 Light Curing are suitable for bonding as they fulfill better clinical acceptability shear bond strength (SBS) in comparison to the conventional light-cured orthodontic bonding adhesive (Transbond XT, 3 M Unitek) (Uysal et al. 2009). In another study, Bishara et al. compared the shear bond strength between Grandio (Voco, Germany) and Transbond XT, 3 M Unitek, while testing the bonding of the orthodontic brackets. It was found that the nanofilled composites demonstrated better bonding ability, nice flowability, and ready attachability in orthodontic bracket base (Bishara et al. 2007). Over the past decade there has been an increased demand in manufacturing aesthetic orthodontic wires that would complement tooth colored brackets. Responsive polymer materials are those polymers that can adapt to surrounding environments, regulate

transport of ions and molecules, change wettability and adhesion of different species on external stimuli, or convert chemical and biochemical signals into optical, electrical, thermal, and mechanical signals and vice versa (Stuart et al. 2010). The sharp memory polymers can memorize a macroscopic or equilibrium shape and then be manipulated and fixed to a temporary or dormant shape under specific conditions of temperature and stress. At later phase they can relax to original, stress-free condition under thermal, electrical, or environmental condition which is associated with elastic deformation stored during prior manipulation toward its equilibrium shape accompanied by an adequate and prescribed force. The sharp memory polymers have the ability to meet the unattainable needs of current orthodontic materials which will further comfort the procedure of orthodontist. Once placed inside the mouth, these polymers can be activated by body temperature or light and enable the tooth movement and provide lighter more constant forces giving less pain to patients. The sharp memory polymeric materials are colorless and stain resistant which give aesthetically appealing appliance during treatment. Moreover the high percent of elongation of sharp memory polymers provides continuous force for a long range of tooth movement hence less number of visits to patients (Redlich et al. 2008). Using these properties and better features, aesthetic nanocomposite wires can be formed for orthodontic purpose (Meng and Hu 2009; Leng et al. 2011).

These findings suggest that in the future, orthodontic nanorobot-based treatment could directly manipulate the periodontal tissues, allowing rapid and painless tooth straightening, rotating, and vertical repositioning within minutes to hours. With this method, the need for the cumbersome and dreaded braces could be eliminated.

Diagnosis of Oral Cancer

The noticeable contribution from nanotechnology in the field of dentistry is mostly observed in the diagnosis of oral cancer. Obtaining the image plays a critical role in designing of treatment plan and overall cancer management. For getting basic information regarding tumor location, size, and spread, standard structural imaging techniques such as computer tomography (CT), molecular resonance imaging (MRI), and ultrasound are used. However, these imaging techniques become less reliable to distinguish between benign and metastatic tumor where the tumor size is smaller than 5 mm (Popovtzer et al. 2008; Reuveni et al. 2011). Recently gold nanoparticle (AuNP)-based CT imaging have been shown progress. For specificity these gold nanoparticles can be functionalized with ligands to target the receptors of overexpressed tumors (Hainfeld et al. 2004; Marega et al. 2012).

One of the most common types of cancer in oral oncology is the oral squamous cell carcinoma, representing ~6% of all cases of cancers. Selective targeting of oral squamous cell carcinoma is an enduring problem because of the lack of specificity of current drugs. Popovtzer et al. (2008) studied the molecular CT imaging of oral cancer with the targeted gold nanoparticles. They reported that the attenuation coefficient is five times higher than the untargeted cells of oral squamous cell carcinoma.

Yang et al. (2011) conjugated cell-penetrating peptides to near-infrared quantum dots for cancer diagnostics. In their pioneering approach, the group labeled oral squamous cell carcinoma cells with quantum dots and conjugates by endocytosis for visual in vivo imaging on a mouse model.

In another study, Bhirde et al. (2009) demonstrate superior efficacy of single wall carbon nanotube bioconjugate, over nontargeted bioconjugates. The three colors, two-photon intravital video imaging demonstrated administration of epidermal growth factor (EGF) targeted single wall carbon nanotube quantum dot in live mice which was selectively taken up by head and neck squamous carcinoma cell (HNSCC) tumors, whereas the nontargeted single wall carbon nanotube quantum was cleared from the tumor region in less than 20 min. Regression in tumor growth was fast in mice treated with single wall carbon nanotube–cisplatin–EGF compared to nontargeted single wall carbon nanotube–cisplatin.

The above studies suggest that the nanotechnology is not only restricted to the tooth restoration, rather it has the potential in the imaging of oral cancer as well. Also imaging with the nanotechnology can help to diagnose and combat the oral cancers during initial stages.

3.2.2 Application of Nanotools in Preventive Dentistry

The aim of modern dentistry is prevent the disease progression before it enters in the noncontrollable state. The prevention of tooth decay and treatment of lesions and cavities are the prime concerns; thereby treating dental diseases with the help of nanotools has been of great interest (Hannig and Hannig 2010). Application of nanotools for preventive dentistry can be stretched from dentin hypersensitivity to drug delivery and tissue engineering.

Dentin Hypersensitivity

Dentin is a yellow-hued calcified tissue and the major components of teeth usually covered by enamel on the crown and cementum on the root that surrounds the entire pulp. It has elastic-like properties and serves as the support for enamel. Due to its soft nature, it decays rapidly and form cavities when not treated properly. The dentin hypersensitivity occurs when the protective layers are removed causing pain (Addy and Urquhart 1992). The protective layer gets lost due to the response to chemical, thermal, or osmotic stimuli or any other form of dental defect or pathology. Apart from that, improper tooth brushing, gingival recession, and periodontal also expose the root surfaces, and the thin covering cementum layer (approximately 20–50 μm) is easily lost. In all these situations, the sensitive dentin tissues are exposed which has numerous tubules that in turn changes the fluid pressure hydrodynamics of the fluid inside the dentinal tubules and is subjected to many external sources of irritation. Liu et al. (2007) explored the possibility of nanoscale gold particle in the

occlusion of dentinal tubules that would reduce movement of dentinal fluid or dentin permeability should decrease sensitivity. The gold nanoparticles (GNPs) possess smaller dimension than dentinal tubules, so they can easily enter the tubules without much resistance. Also the GNPs have large absorption coefficients (Taton et al. 2000; Liu et al. 2007). The dentinal tubules were occluded with GNPs by adsorbing onto the inner dentinal tubule walls, followed by a silver staining to reduce the dentin sensitivity. In another process, highly concentrated GNPs are brushed into the opened ends of the dentinal tubules and after laser irradiation induced photofusion of GNPs via photothermal conversion (Liu et al. 2007). Using biological materials' induction of dental nanorobots offers a speedy cure to dentin hypersensitivity by precisely occluding the tubules in minutes (Schleyer 2000).Based on the above discussion, it can be concluded that owing to the small size, the nanoparticles have the capacity to enter the dentinal tubules and treat the dentin hypersensitivity. Although nanoformulation-based products are not yet available in the market for dentin hypersensitivity, in the near future, this potential can be commercialized.

Drug Delivery and Tissue Engineering

Tissue engineering has become one of the promising approaches for delivering of therapeutically relevant molecules to treat the damaged tissues. These molecules can be effectively loaded in large quantities in scaffolds or nanoparticles for sustained and controlled release. The nanochemistry of the scaffold can carry signaling molecule for homing and the therapeutic molecule for sustained release to the surrounding tissues for regulation of cellular function (Treuel et al. 2013; Kettler et al. 2014). Various polymeric materials or inorganics and their hybrids have been developed for the delivery of molecules. In case of treatment of periodontal disease, two methods are used: antibacterial process to avoid progression of the disease and the regenerative therapy which can be able to regenerate the damaged tissue (Pragati et al. 2009). For the regenerative treatment, bone morphogenic proteins (BMPs) are used as they are involved in osteogenic differentiation of stem cells are for the regeneration of periodontal ligament (PDL), cementum and alveolar bone (Giannobile et al. 1998; Ripamonti et al. 2001; Wikesjö et al. 2004). Similarly in case of dental pulp infection, successful regeneration of odontoblasts, endothelial cells, fibroblasts, and even neurons is greatly needed to restore pulp tissue. In the regeneration process, different growth factors such as transforming growth factor (TGF-β1), bone morphogenic protein (BMP), and platelet-derived growth factor can be either used alone or in combination. For periodontal regeneration, PLGA nanoparticles were developed which can encapsulate minocycline for periodontal infections in a sustained manner for several weeks impacting significant antibacterial effect compared to native (Kashi et al. 2012).

Therefore it can be concluded that nanotechnology can play a major role in the development of cost-effective therapies for drug delivery and in situ tissue regeneration.

3.2.3 Applications of Nanotools in Operative Dentistry

Operative dentistry can be defined as the branch of dentistry which deals with the diagnosis and treatment of tooth defects which do not require full restoration. Thereby operative dentistry is of prime importance for enhancing the dental health. Employment of nanotechnology for operative dentistry is the budding field wherein various nanotools can be explored. Also various commercial preparations of the dental materials are available and are summarized in Table 3.1. In general, up till now nanotools in the form of nanocomposites, antimicrobial agents, root-end sealants, and dental biofilm penetrators have been instigated.

Nanocomposites

Dental composites are the solid materials composed of synthetic matrix, inorganic fillers, initiators, activators, and coupling agents. The activator promotes the light-initiated polymerization of the organic matrix to generate cross-linked polymer structure, and coupling agents mainly with silane groups ensure the bonding of nanofillers to the polymer matrix.

The most commonly used organic matrix in the commercial dental composites is Bis-GMA monomer. As high viscosity-related problems are associated with the Bis-GMA, it must be diluted with other fluid monomers to attain the desired viscosity for dental composites. Other base monomers used in manufacturing of commercial composites are uretane dimethacrylate (UDMA), triethyleneglycol dimethacrylate (TEGDMA), ethoxylated bisphenol-A-dimethacrylate (Bis-EMA), urethane tetramethacrylate (UTMA), bis(methacryloyloxymethyl) tricyclodecane, and decanediol dimethacrylate (D3MA) (Ferracane 1995).

Table 3.1 Various marketed preparations of dental materials

Dental approach	Marketed preparations
Orthodontic materials	Ketac ™ N100 Light Curing, Filtek Supreme Plus Universal, Grandio (Voco, Germany), Transbond XT; 3 M Unitek, Adper™ single bond plus, Sandvik Bioline RK 91™
Nanocomposites	Filtek Supreme (3 M ESPE, St. Paul, MN, USA), Premise (Kerr/Sybron, Orange,CA, USA) Nano-DCPA whiskers, TTCP-whiskers, Polymer kaolinite
Root-end sealant	Endo Sequence BC sealer, Gutta-Flow Sealer
Plasma-based nanotools	MicroPlaSter
Periodontal bone grafts and bone replacement material	Ostim (Osartis GmdH, Germany), NanOSST(Angstrom, Medica, USA), Vitoss (Orthovita Inc., USA)

When the inorganic phase of dental composite is nanosized, it becomes nanocomposites. Nanofillers in the nanocomposites are added either in dispersed form or in cluster form. Due to their smaller size dimension, the nanofillers are invisible and are capable to improve the optical property (Mitra et al. 2003). Owing to their small particle size, nanofillers are capable to increase the overall filler levels. Also the physical properties of nanocomposites can be improved by increasing and decreasing the filler level and resin content, respectively.

In dental composites, good mechanical properties can be obtained by strong covalent interaction between the fillers and organic matrix. Coating of fillers with silane coupling agent assures the bonding between the two phases, i.e., matrix and fillers. 3-Methacryloxypropyltrimethoxysilane.

(MPTS) is a typical example of coupling agent. One end of this molecule can be bonded to the hydroxyl groups of silica particles and the other end copolymerize into the polymer matrix.

Various nanocomposites are available in the market. Filtek Supreme (3 M ESPE, St. Paul, MN, USA) is a dental nanocomposite based on nanomers and nanoclusters. Nanomers are the uniformly dispersed, nonaggregated, and nonaglomerated silica particles of 20–70 nm particle size. In the Filtek Supreme, filler content is about 58–60% by volume and 78.5% by weight (Mitra et al. 2003).

Premise (Kerr/Sybron, Orange, CA, USA) is another dental nanocomposite, composed of three different types of filler components such as nonagglomerated silica nanoparticles, prepolymerized fillers (PPF), and barium glass fillers. The combination allows the filler loading up to 69% by volume and 84% by weight (Bauer et al. 2000).

Dental nanocomposites can be mechanically strengthened by incorporation of reinforced fillers such as nanofibers (Tian et al. 2007), short E-glass fibers (Garoushi et al. 2008), and TiO2 nanoparticles (Xia et al. 2008). Dental nanocomposite of ion-releasing properties have been developed to increase the mineral content and to control the dental caries. Nanocomposites such as nano-DCPA whiskers (Xu et al. 2007) or TTCP-whiskers (Xu et al. 2009) and polymer kaolinite (Wang et al. 2007) release calcium, phosphorus, and fluorides, respectively, thereby increasing the mineralization of tooth and control the caries development. It has been proven that the significant improvement in the field of dental materials has been majorly contributed by the development of dental nanocomposites.

Potential of nanotechnology has been extended to the characterization of dental materials as well. Various techniques such as atomic force microscopy, scanning electron microscopy, X-ray photoelectron spectroscopy, and Piezoresponse force microscopy (PFM) have been successfully utilized to study the surface characteristics of dental nanocomposites (Sharma et al. 2010; Khanal et al. 2015; Salerno and Diaspro 2015). Figure 3.3 enlists the various techniques for the nanoscale characterization of the dental materials.

Nano-characterization of dental materials	
Atomic force microscopy (AFM)	Optical profilometry
Scanning electron microscopy (SEM)	X-ray diffraction spectroscopy
Scanning tunneling microscopy (STM)	Fourier-transformed infrared spectroscopy (FTIR)
X-ray photoelectron spectroscopy	Raman scattering
Dynamic mechanical analysis (DMA)	Energy dispersive spectroscopy (EDS)
Transmission electron microscopy (TEM)	Total internal reflection fluorescence (TIRF)
Optical microscope mapping	High speed-AFM
Lorentz contact resonance spectroscopy	Fluorescence resonance energy transfer (FRET)
Piezoresponse force microscopy (PFM)	Scanning ion conductance microscopy (SICM)
Scanning probe microscopy (SPM)	Live-cell interferometry (LCI)

Fig. 3.3 Nanocharacterization techniques of dental materials
Various techniques to characterize the surface morphology and to study the surface behavior of dental materials are enlisted

Nanoparticles as Antimicrobial Agent

The plethora of work in the field of design and development of nanoparticles has led to an enormous use of these systems for treatment of versatile kinds of diseases including dental infections and other dental afflictions. Such usage of nanoparticles is akin to the Paul Ehrlich's concept of "magic bullet" where the systems invariably engage with the target and yield positive therapeutic outcomes. Owing to the poly-cationic or anionic nature and large surface area with charge density, nanoparticles interact effectively with the bacterial cell and result in higher antibacterial activity (Allaker 2010). Chitosan nanoparticles being antimicrobial and biocompatible in nature have been exploited in the treatment of bacterial biofilm and wound healing (Raafat and Sahl 2009; Kong et al. 2010). Chitosan nanoparticles are less cytotoxic in nature and can be used as drug delivery carriers in various systemic diseases. They provide a remarkable improvement in root canal disinfection by selectively removing the residual adherent and nonadherent bacteria also increasing the flux of antimicrobial agent from the root canal sealant (Shrestha et al. 2009). The nanosize hydroxyapatite particles having remineralization capacity also have been shown to

inhibit the formation of oral biofilm (Venegas et al. 2006). Various oral healthcare products such as toothpastes and mouth rinses containing nanohydroxyapatite have been developed. And it has been suggested that their efficacy is related to the size specific effects of nanohydroxyapatite (Reynolds et al. 2003; Rahiotis et al. 2008).

Also nanotechnology is gaining tremendous popularity in the present world due to its capability of modulating metals into nanosize, which enormously modulates the physicochemical and optical properties of metals. Silver nanoparticle which is the derived form of metallic silver is a good example of antimicrobial agent. Also, as the bacterial resistance toward the antibiotics is increasing day by day, then in such cases, the use of silver nanoparticle can be an alternative. Various medical applications of silver nanoparticles are available as silver-based dressings, silver nanogels and nanolotions, etc. The antimicrobial efficacy of silver, gold, and zinc nanoparticles against the bacterial strain of *S. mutans*, which is a major causation of dental caries, was compared. The studies revealed higher antimicrobial effects against *S. mutans* of silver nanoparticles at lower concentrations than gold or zinc (Hernández-Sierra et al. 2008). Also it is concluded that with size reduction, contact surface increases, which is important for the broad-spectrum antimicrobial effect of silver. The low concentration of silver can avoid the teeth staining. In this manner silver nanoparticles can be a powerful approach for treatment of dental caries.

Nanotools as Root-End Sealant

With the enhancement of nanotechnology, the quality of dental biomaterial has also improved. Nanotechnology manufactures materials with better properties or modulating the properties of existing materials. Root-end sealants play the important role as pulp filling material in a carious primary tooth. Also the nanomaterial enhanced retrofill polymers (NERPs) impart superior strength and adaptability to the tooth surface. In the study of extracted tooth model, the results showed that NERP materials reduce the microleakage and hence have the ability to seal effectively. Bioaggregate white nanoparticles are a novel type of ceramic cement primarily which consists of calcium silicate, calcium hydroxide, and hydroxyapatite (Yuan et al. 2010). Recently, Endo Sequence BC sealer which is a bioceramic-based nanomaterial has been developed. It mainly consists of calcium silicate, calcium hydroxide, calcium phosphate, zirconia, and a thickening agent. Handling and physical properties of the nanoparticles have been improved. During the root canal treatment, hydration reaction occurs with the formation of calcium silicate and hydroxyapatite. Availability of water is the key factor for hydration reaction and setting time in overly dried canals. Nanosized particles assure delivery of capillary needle of size from 0.012 to adopt into the irregular dentin surfaces. Within few hours, it sets hard and provides excellent seal with dimensional stability. This form of hydroxyapatite is bioactive and biocompatible. Its high alkaline pH, i.e., 12.8, provides additional antimicrobial properties as well. Similarly Gutta-Flow Sealer is an example

of root-end sealant which consists of silicon, gutta-percha powder, and silver nanoparticles (Koch and Brave 2009). This nanosealer sets within half an hour and has good biocompatibility and dimensional stability. It is reported that this material can provide better bacterial penetration resistance and enhance the sealing capability. Also from the infection reference, antimicrobial activity of root-end sealants can be a synergistic effect. Recently antibacterial nanoparticle, i.e., quaternary ammonium polyethyleneimine (QPEI), was incorporated into already existing sealers like AH plus, Epiphany and Guttaflow. Results suggest prolonged antibacterial activity without compromising the mechanical properties. In order to obtain antibacterial effect in endodontic sealers, 0–2% nanoparticles of QPEI were incorporated into the marketed sealers. The obtained product was very stable and remains biocompatible; however the antibacterial effect was excellent (Abramovitz et al. 2012).

The above studies suggest that the nanosize-based root-end sealants are better in strength and durability than the other root-filled materials. Also with the nanobased root-end sealants, antibacterial effect can be obtained, and this will further aid to the root canal treatment.

Nanotools for Dental Biofilms

Nanotechnology tools help to study the interspecies interaction involved in the development of biofilm and can be used to understand the demineralization/remineralization process in development of dental caries. For example, atomic force microscope can be used to detect bacterial plaque-generated demineralization at an ultrasensitive level.$^{16}O/^{18}O$ reverse proteolytic labeling is another application of nanotechnology used to determine the influence of bacterial culture on the cell envelope proteome of *Porphyromonas gingivalis* which is responsible for periodontitis (Ang et al. 2008). Nanotechnology also makes it possible to detect both cultivable and non-cultivable bacteria and can selectively and preferentially remove cariogenic bacteria without disturbing the normal oral flora. Similarly plaque acidity, which is a good marker of tooth demineralization, can be monitored using a microscale planar pH sensor. New silver-based nanotechnology has been proven to be active against biofilms. The silver has high affinity for negatively charged side groups like sulphydryl, carboxyl and phosphate moieties of bacterial cell wall. Silver selectively binds to these moieties and arrests the bacterial cell wall synthesis, protein function, membrane transport, electron transport, and other physiological cell functions. It is effective against biofilm-associated pathogens including *E. coli, S. pneumoniae, S. aureous*, and *A.niger* (Bhardwaj et al. 2009). For preventing cell growth in certain bacteria, as little as one part per billion of silver may be effective (Gibbins 2003). The various aspects of nanotechnology in oral biofilm have led us to visualize the smart mouthwash comprises of nanomachines which will selectively allow the nonpathogenic flora of mouth to flourish in healthy environment and simultaneously inhibits the pathogenic ones, thereby increasing the oral health of human beings.

3.2.4 Applications of Nanotechnological Tools in Periodontal Therapy

Periodontal disease is a complex disease involving the destruction of tooth supporting materials and alveolar bone loss (Verma et al. 2010). Thus the ultimate goal of therapy is to restore the tissue destruction by repair and regeneration. Similar to the other branches of dentistry such as preventive, restorative, and operative, periodontics is also impacted by the advances of nanotechnology. Utilization of nanotechnology or nanotools for periodontal therapy has led to the development of better imaging and drug delivery alternatives and mainly includes the laser plasma-based nanotools, nanobone fibers, periodontal bone grafts, and nanomaterials for periodontal drug delivery.

Laser Therapy and Plasma-Based Nanotools

Lasers in the range of middle- and far-infrared regions allow their successful use in dentistry for hard and soft tissue procedures, because of its high sensitivity and the lack of the associated risks of ionizing radiation (Jha et al. 2017). For periodontal applications, lasers have been explored in various procedures such as soft tissue extraction, calculus removal, bacterial reduction, incision and ablation, biostimulation, decontamination of root and implant surface, and bone removal. Laser wavelength like Er:YAG, Er, and Cr:YSGG are greatly absorbed by hydroxyapatite and can be utilized for bone removal (Romanos 2015).

Laser therapy (diode and Nd:YAG lasers) can be of great help in treating noninvasive problems like gingival hyperpigmentation and also revealed that it could easily replace the need for analgesics (Shankar et al. 2013). Low-temperature plasma has a promising usage in the field of dentistry and can be effectively used to sanitize the gingival crevices and periodontal pockets (Chen Fa-Ming et al. 2009). This type of treatment alleviates the fear of dental visits which is called as odontophobia and can help in the treatment of dental infections in children and adults as well.

Plasma devices could be highly effective in inhibiting bacterial growth significantly in the root canal and consequently lead to complete sterilization during dental treatments (Liang et al. 2015). A significant advantage of plasma is amenability to both wet and dry environments, and henceforth, presence of blood, gingival crevicular fluid, and saliva does not compromise its efficacy (Jha et al. 2017). They can regenerate and differentiate periodontal stem cells and thus show potential as a future dental therapy, and they facilitate successful gingival treatments such as that for gummy smile, resulting in the more rapid generation of various dental-related cells (e.g., fibroblasts and collagen) with the least amount of postoperative pain to the patient (Miletić et al. 2013). Plasma is available in various types like dielectric barrier discharge, plasma jet, plasma torch, and barrier coronal discharge. Research is being done in the research centers and medical industry with advent of new devices for fast and easy treatment. In medical field, argon plasma torch, i.e.,

MicroPlaSter, has been introduced for a randomized controlled trial for patients with chronic infected wounds with well-tolerated healing results. Summarily, plasma-aided dental devices that are futuristic in its approach may replace exciting technologies and emerge as a future nonsurgical, noninvasive treatment modality, especially in periodontal dentistry.

Nanobone Fibers

Nanofibers are the preferred reinforced constituent of dental nanocomposites. In order to enhance the mechanical strength of nanocomposite, nanofibers are incorporated into them. Nanobone fibers have 100 times the strength to that of steel (polyphosphazene nanofibers). They have attained popularity in local drug delivery system due to their superior properties (Slavkin 1999). Studies suggest that incorporation of high-strength inorganic fibers in the dental composite led to the significant improvement in mechanical properties (Fong 2004; Callaghan et al. 2006) Recently, nanofibers have been employed to formulate HA- and fluoro-HA-containing ceramics (Kim and Kim 2006). Nanofibrillar silicate crystals have also been studied for the capacity of regenerating or supporting of dental structures. One such type of combination consists of 2,2′-bis-[4-(methacryloxypropoxy)-phenyl]-propane (Bis-GMA) and thinning agent as triethylene glycol dimethacrylate (TEGDMA) (Tian et al. 2007; Tian et al. 2008). After incorporation of relatively small amount of nylon 6 nanofibers into the resin, the three-point bending test of the modified dental composite suggests that flexural strength, work of fracture, and elastic modulus were considerably increased. But addition of more than 6% mass fraction of nylon 6 nanofibers into the resin did not enhance the mechanical properties of the dental composite significantly. If formulated in the correct proportions and with uniform distribution, nanofibers were reported to enhance the physical properties of the composites.

As the size of the nanofiber is less than the wavelength of visible light, it does not affect the transparency of the nanocomposite and offers an advantage of using nanofibers as reinforcement materials (Bergshoef and Vancso 1999). The aforementioned advantages of polymer nanofibers make them a promising candidate for future development of orthodontic composites that are of sufficient mechanical strength and of desired aesthetic properties.

Periodontal Bone Grafts and Bone Replacement Material

In dentistry, one of the biggest challenges is the predictable regeneration of alveolar bone destroyed by periodontitis. A great success in this field has been achieved by the periodontal bone grafts (Brunsvold and Mellonig 1993). The periodontal bone grafts are inserted with the objectives of reduction of probing depth; regain the clinical attachment, alveolar bone fill and regeneration of bone, cementum and periodontal ligament (Schallhorn 1977). Periodontal bone grafts allows ideal bone

regeneration as they have greater surface area as compared to other synthetic bone grafting material, due to their microporosity and nanoporosity (Slavkin 1999). In bone therapy, from very recent time autogenous and allogenic bone grafts have been used, synthetic biomaterials have been developed, but none of them is similar to the natural bone in terms of structure and composition. Owing to the fact that hydroxyapatite/collagen systems are structure- and composition-wise similar to the natural bone, their nanocomposites are promising bone grafts. Their functional characteristics in the nanorange facilitate collagen growth and subsequent periodontal tissue formation (Whitesides and Love 2001). Smart material for periodontium, developed through nanotechnology, will aid in repair and regeneration of bone (Khosla 2009). Synthetic hydroxyapatites have been marketed in various forms such as resorbable, solid nonresorbable, and porous nonresorbable. Ostim (Osartis GmdH, Germany), NanOSST(Angstrom, Medica, USA), and Vitoss (Orthovita Inc., USA) are the hydroxyapatite nanoparticle used to repair bone defects (Sheikh et al. 2015). Other synthetic variant is the bioglass. Bioactive glass is mainly composed of sodium calcium salts, phosphates, and silicon dioxide (Anderegg et al. 1999). Bioactive glass possesses the ability to release mineral ions and promote natural bone regeneration. Once it reacts with blood, it attaches to the bone and slowly releases silica ions (Hoppe et al. 2011). There it initiates osteoblast differentiation and proliferation (Hench 2006; Jones 2015). In due course of time, it gets fully absorbed and replaced by the bone. When the bioactive glass is mixed with autogenous bone graft material, synergistic effect is produced, and natural bone regeneration process gets doubled (Oonishi et al. 2000). In summary the complex procedure of bone regeneration can be minimized with the various types of available bone grafts and bone replacement materials.

Nanomaterials for Periodontal Drug Delivery

Modern delivery systems are designed with the purpose of targeted and controlled drug release. Up to now polymeric or microparticulate delivery systems are utilized in dentistry which controlled the drug release due to their structure, but the intensive research in the nanotechnology leads to development of nanomaterials for effective periodontal drug delivery. When compared with the microparticulate systems, the nanomaterials offer several advantages such as high dispersibility in aqueous medium, controlled drug release, and better stability. Also owing to the small size, nanomaterials can penetrate deep inside the periodontal pockets and are suitable candidate for periodontal drug delivery (Jain et al. 2008). Nanomaterials particularly nanospheres, core-shell structure, nanotubes, and nanocomposites have been widely employed for controlled drug delivery system (Jain et al. 2019). Nanospheres can be fabricated with biodegradable polymer and drug for the controlled drug delivery. Biodegradable nanoparticles formulated with polyethyleneglycol dimethacrylate (PEGDMA) and 2-hydroxyethyl methacrylate (HEMA) can be used as drug delivery carrier for periodontal applications. Further these nanoparticles can be suitably incorporated into hydrogel matrix for ease of application (Bakó et al. 2007).

Recently, Pinon-Segundo et al. (2005) formulated and characterized triclosan-loaded nanoparticles through the process of emulsification-diffusion so as to obtain a novel delivery system directed toward periodontitis. These nanoparticles were formulated using poly(D,L-lactide-coglycolide), poly(D,L-lactide), cellulose acetate phthalate, and polyvinyl alcohol which were taken as stabilizer. These nanoparticles act as a homogeneous polymer matrix-type delivery system in which triclosan was dispersed. Results indicate that triclosan nanoparticles were capable to reduce inflammation at the experimental sites (Pinon-Segundo et al. 2005). The ongoing research in nanotechnology suggests that the suitably developed and optimized nanoformulations or nanomaterials could be effective drug delivery carriers for the periodontotherapy.

But before the submission of the formulation for patient use, it is imminent that these are subjected to animal studies for which number of authors has devised various experimental models, and the summary of the work is enlisted in Table 3.2.

3.3 Barriers of Nanotechnology

Continuous improvements of traditional approaches, development of novel restorative materials, advanced medications, and pharmacological strategies will continue to improve dental care. Derivatives of nanotechnological tools like nanoparticles and nanotubes have significant role in operative dentistry, periodontal management, endodontics, and restorative dentistry. Nanotechnology is set to revolutionize clinical dental practice. In no distant future, oral healthcare services will become less stressful for the dental surgeons, more acceptable to patients, and the outcome will significantly become more favorable. Optimal utilization of the advantages and opportunities offered by nanotechnology in clinical dental practice will facilitate improvements in oral health. The misuse and abuse of any technology continues to be a human issue, which could not be easily discerned by even intelligent systems**.** Nanotechnological tools if not properly controlled and directed carry a significant potential for misuse and abuse. The rapid progress and proper investigation will ensure that the development which seems unbelievable today is possible in the near future. However, in nanorobot mass production technique, precise positioning and assembly of molecular-scale part require simultaneous coordination of activities of large numbers of independent micron-scale robots. If the precision fails to some extent, then it will be havoc for the patients; hence it needs to be taken care of. Apart from that, the biocompatibility issue of the materials used till now or that will be engineered in the near future should possess no toxic effect to the health. Last but not the least, the funding and strategic issues because of inadequate venture capital, excessive bureaucracy and lack of medical input, insufficient integration of clinical research, and inefficient translation of concept to product would raise social issues of public acceptance, ethics, and regulation required for human safety.

Table 3.2 Animal models of periodontitis to assess the in vivo behavior of dosage forms

Animal model	Dosage forms	Aim of study	References
Mouse	Local delivery system	To study the effect of locally delivered antimicrobial agent on the inflammatory response	Vanderkerckhove et al. (1998)
Rat	Topical	To assess the potential effectiveness of the developed formulation in treating periodontitis	Luan et al. (2008), Jain et al. (2020)
	Gel foam pellet	To evaluate the combined efficacy of locally delivery of alandronate and tetracyclines in reducing alveolar bone loss	Yaffe et al. (2003)
	Isosorbide gel	To evaluate the role of nitric oxide (NO) on bone metabolism and effect of isosorbide on periodontal disease	Leitao et al. (2004)
	Bioerodible polymer insert	To evaluate a new class of bioerodible polymers as periodontal inserts for controlled release of metronidazole	Gates et al. (1994)
Beagle dogs	Nanoparticles	To evaluate in vivo efficacy of the developed dosage form of triclosan	Pinon-Segundo et al. (2005)
	Gel	To evaluate the potential of locally injected simvastatin in human sized periodontal defects	Morris et al. (2008)
	Collagen gel	To examine the effects of bFGF on the regeneration of cementum and periodontal ligament in experimentally induced partial defects	Sato et al. (2004)
	Ointment	To evaluate clinical, enzymatic and microbiological effects of controlled release localized administration of minocycline on dogs with periodontitis	Hirasawa et al. (2000)
	Biodegradable membrane	To evaluate the regenerative effect of a 25% doxycycline loaded biodegradable GTR membrane	Chang and Yamada (2000)
	Film-forming solutions	To evaluate the in vivo efficacy of the developed formulation	Kozlovsky et al. (1992)
	Dental paste	To study the effect of topical metronidazole therapy on ligature-induced periodontitis	Klinge et al. (1992)

3.4 Conclusion

The advancements in nanosciences/nanotechnology have led to an unprecedented growth in niche areas like medicine and its allied fields. Of these, an insurmountable effect has been on the contemporary dental practice and has led to the emergence of a newer discipline of dentistry. The bottom-up and top-down approaches of nanotools in dentistry aims at circumvention of all diseases of bony origin, dental adnexa, and associated soft tissues. One of the major challenges of dentistry is to address afflictions located at difficult to reach sites in the oral cavity. These often demand precise and controlled handling and require scrupulous sterilization so as to reduce bacterial deposition. Henceforth, the advents of laser, plasma, dentrifobots, and nanoassemblers have added robustness to dental cleaning and better oral hygiene. Likewise, the nanoencapsulation of antibiotics and their delivery into periodontal pockets ensure drug availability above MIC's over a prolonged period of times and do not require large doses of oral antibiotics. dentin hypersensitivity, tooth repairs, caries filling, and diagnosis of oral cavity cancer are other areas wherein the nanotools are highly commendable. Optimal utilization of the available nanotools in modern dental practice will definitely promise better oral health with a markedly reduced odontophobia.

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Chapter 4
Nanopharmaceuticals: Synthesis, Characterization, and Challenges

Sunita Ojha, Dharitri Saikia, and Utpal Bora

Contents

Abstract Nanomaterials have a great potential in pharmaceutical industries for drug delivery, gene delivery, gene therapy, tissue engineering, diagnosis, and in vivo imaging. For this purpose, polymeric nanoparticles, lipid-based nanoparticles, carbon-based nanoparticles, and inorganic nanoparticles are explored. The various syn-

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thesis methods of nanopharmaceuticals are broadly categorized into top-down and bottom-up approaches. Top-down approaches include mechanical milling, electro-expulsion, and sputtering techniques in which bulk materials are broken into nano-sized particles. While in bottom-up approach, nanomaterials are synthesized by atomic level self-assembly. Chemical reduction and green synthesis of nanomaterials are two examples of bottom-up approaches. The properties of nanomaterials such as morphology, structure, hydrophobicity, purity, drug release properties, and toxicity are characterized by several biophysical techniques such as spectroscopy, scattering techniques, imaging through electron microscopy, mammalian cell culture, and animal studies. Synthesizing a nanopharmaceutical of desired characteristic and activity is challenging in every step. Consistency and reproducibility are the major challenges which need to be addressed before considering any nanopharmaceutical to be a potential lead.

Keywords Nanomaterials · Nanopharmaceuticals · Polymeric nanoparticles · Lipid-based nanoparticles · Carbon-based nanoparticles · Inorganic nanoparticles

4.1 Introduction

The term “nanopharmaceuticals” refers to the convergence of nanotechnology and pharmaceuticals. Nanotechnology is used to define product, processes, and properties at nanoscale. Reducing the material size to 1–500 nm in at least any one of the three dimensions results into change in their physical, optical, chemical, and biological properties. These properties are unique and different from their bulk counterparts due to high surface area to volume ratio. These unique properties are explored in drug delivery, therapeutics, and diagnosis. Drug delivery at the target site in correct dose is a challenge for most of the traditional drugs. Hence, drugs are administered at higher doses which increase side effects. Further, traditional drugs are not capable of crossing blood–brain barrier and blood–retinal barrier due to their anatomical features. Nanotechnology-based drugs with improved therapeutic properties and precision in drug delivery at target site will minimize the drug dosage. Nanopharmaceuticals have shown to cross intact blood–brain barrier and blood–retinal barrier. The ability of nanopharmaceuticals to permeate the tight epithelial junctions of skin is utilized in topical emulsions. Nanotechnology-based drug formulations also improve their pharmaceutical properties, solubility and permeability across intestinal epithelium. Drugs encapsulated in nanomaterials are protected from degradation (enzymatic and nonenzymatic), complexation (with chelating ligands and metal ions), and intestinal efflux (Rodriguez-Fragoso et al. 2014). Nanopharmaceuticals also alter the pharmacokinetic properties of the drugs such as (1) in vivo improved drug release profile, (2) enhanced drug absorption, (3)

site-directed drug distribution, (4) modified drug metabolism pattern, (5) prolonged drug residence time in body (e.g., in blood circulation), and (6) delayed and/or decreased renal excretion of the drug (Hamidi et al. 2013).

Nanotechnology-based biosensors enable early disease detection due to high sensitivity and specificity. Detection of specific genetic sequence in a sample can be performed using short fragments of DNA tagged with gold nanoparticles. Gold nanoparticles have also made gene sequencing efficient. Better understanding of molecular basis of diseases and their prevention is possible through nanotechnology (Nikalje 2015).

Although nanotechnology is an age old concept, its application in healthcare industries is new and needs to be constantly researched and improvised. The evolution of nanotechnology in healthcare industries can be visualized from the number of nanotechnology-based therapeutics being approved by FDA for clinical use. Two dozen nanotechnology-based drugs have been approved by FDA, and more are under clinical trials (Shi et al. 2010). Nanopharmaceuticals such as Doxil® and Abraxane® are already available in market (Devalapally et al. 2007). Some FDA-approved nanopharmaceuticals are listed in Table 4.1.

Nanomaterials that are explored in pharmaceutical industries include polymeric nanoparticles, liposomes, magnetic nanoparticles, carbon nanotubes, dendrimers, carbon dots, metallic nanoparticles, etc. Therefore, these nanopharmaceuticals are categorized into organic and inorganic nanopharmaceuticals. Nanopharmaceuticals are synthesized by physical, chemical, and biological methods and characterized by several biophysical techniques. The properties of nanopharmaceuticals are affected by local environment and exposure duration. During large-scale production of nanopharmaceuticals, consistency and reproducibility are the major challenges. Further, removal of residual organic solvent to ensure purity and safety of the nanopharmaceuticals is a tedious task. Therefore, to overcome these challenges, upgradation of the techniques involved in nanopharmaceutical synthesis and characterization is required. In this chapter, synthesis of both organic and inorganic nanostructures is described along with their characterization techniques and challenges faced during their development.

4.2 Synthesis of Nanopharmaceuticals

Nanopharmaceutical synthesis techniques are categorized into top-down and bottom-up approaches. Top-down approach involves breaking a bulk material into smaller pieces mechanically, chemically, or by any other form of energy. In bottom-up approach, atoms and molecules assemble themselves or react chemically and gradually increase in size to form nanostructures. Nanomaterial synthesis method is desirable to have control in manipulating particle morphology, shape, size, and size distribution. Nanomaterials synthesized through self-assembly process allow a good control on these factors (Masala and Seshadri 2004).

Table 4.1 List of FDA-approved nanopharmaceuticals

Sl. No.	Name of the drug	Manufacturer	Type	Target disease	Status	Year of FDA approval
1	Adagen® (pegademase bovine)	Sigma-Tau Pharmaceuticals, Inc.	Synthetic polymer combined with drug/biologics	Severe combined immunodeficiency disease associated with ADA deficiency	Approved	1990
2	Cimzia®	UCB	Synthetic polymer combined with drug/biologics	Plaque psoriasis, psoriatic arthritis, rheumatoid arthritis, Crohn's disease, and other inflammation that may be caused by overactive immune system	Approved	2008–2013
3	Copaxone®	Teva	Synthetic polymer combined with drug/biologics	Relapsing forms of multiple sclerosis (MS)	Approved	1996
4	Eligard®	Tolmer	Synthetic polymer combined with drug/biologics	Prostate cancer	Approved	2002
5	Macugen® (Pegaptanib)	Bausch & Lomb	Synthetic polymer combined with drug/biologics	Age-related macular degeneration	Approved	2004
6	Mircera®	Roche (except in Japan)	Synthetic polymer combined with drug/biologics	Anemia associated with chronic kidney diseases	Approved	2007
7	Neulasta®	Amgen	Synthetic polymer combined with drug/biologics	Postchemotherapeutic infection	Approved	2002
8	Pegasys®	Gentech	Synthetic polymer combined with drug/biologics	Chronic hepatitis B and chronic hepatitis C	Approved	2002
9	PegIntron®	Merck	Synthetic polymer combined with drug/biologics	Hepatitis C and melanoma	Approved	2001
10	Renagel®	GelTex Pharmaceuticals	Synthetic polymer combined with drug/biologics	Chronic kidney disease	Approved	2000

(continued)

Table 4.1 (continued)

Sl. No.	Name of the drug	Manufacturer	Type	Target disease	Status	Year of FDA approval
11	Somavert®	Pfizer	Synthetic polymer combined with drug/biologics	Acromegaly	Approved	2003
12	Oncaspar®	Enzon Pharmaceuticals	Synthetic polymer combined with drug/biologics	Acute lymphoblastic leukemia	Approved	2006
13	Krystexxa®	Horizon pharma	Synthetic polymer combined with drug/biologics	Severe, chronic gout which cannot be controlled by other treatment	Approved	2010
14	Plegridy®	Biogen	Synthetic polymer combined with drug/biologics	Multiple sclerosis	Approved	2014
15	Adynovate®	Baxalta	Synthetic polymer combined with drug/biologics	Hemophilia	Approved	2016
16	DaunoXome®	Galen	Liposome combined with drug/biologics	HIV-associated Kaposi's sarcoma	Approved	1996
17	Marqibo®	Onco TCS	Liposome combined with drug/biologics	Philadelphia chromosome-negative (Ph–) acute lymphoblastic leukemia	Approved	2012
18	Onivyde®	Merrimack	Liposome combined with drug/biologics	Pancreatic cancer	Approved	2015
19	AmBisome®	Gilead Sciences	Liposome combined with drug/biologics	Serious fungal infections and leishmaniasis	Approved	1997
20	DepoDur®	Pacira Pharmaceuticals	Liposome combined with drug/biologics	Postsurgical pain	Approved	2004
21	Visudyne®	Bausch & Lomb	Liposome combined with drug/biologics	Macular degeneration	Approved	2000
22	Doxil®	Janssen	Liposome combined with drug/biologics	Kaposi's sarcoma, multiple myeloma, ovarian cancer	Approved	1995 2008 2005
23	Abelcet®	Sigma-tau	Liposome combined with drug/biologics	Fungal diseases		1995

(continued)

Table 4.1 (continued)

Sl. No.	Name of the drug	Manufacturer	Type	Target disease	Status	Year of FDA approval
24	Curosurf®	Chiesi Farmaceutici	Liposome combined with drug/biologics	Respiratory distress syndrome		1999
25	Estrasorb™	Novavax	Micelle combined with drug/biologics	Menopause-related diseases	Approved	2003
26	Abraxane®	Celgene	Protein nanoparticle with drug/biologics	Breast cancer, lung cancer, pancreatic cancer		2005 2012 2013
27	Ontak®	Eisai Corporation of North America	Protein nanoparticle with drug/biologics	Cutaneous T-cell lymphoma (CTCL)	Approved	
28	Emend®	Merck	Nanocrystal	Postsurgery and postchemotherapeutic symptoms	Approved	2015
29	Tricor®	Lupin Pharmaceuticals	Nanocrystals	To reduce higher cholesterol and triglyceride level	Approved	2004
30	Rapamune®	Pfizer	Nanocrystal	Immunosuppresant (after organ transplant)	Approved	1999

Bobo et al. 2016; Ventola 2017; Anselmo and Mitragotri 2016; Pillai 2014; Hare et al. 2017; Wang et al. 2013

4.2.1 Organic Nanopharmaceuticals

Recently pharmaceutical industry has led the research into organic nanoparticles. Organic nanoparticles contain carbon and hydrogens which form hydrocarbons. Organic nanopharmaceuticals include polymeric nanoparticles, polymeric nanomicelles, dendrimers, lipid based nanoparticles, and carbon-based nanomaterials. The methods used for the synthesis of these nanomaterials are described below.

Polymeric Nanoparticles

The most studied organic strategies for nanomedicines are polymeric nanoparticles (PNPs) of biocompatible and biodegradable nature. PNPs are utilized for targeted drug delivery techniques such as entrapment and conjugation of drugs, prodrugs, stimuli-responsive systems, imaging, theranostics, and controlled distribution of

drugs at tissue and cellular level. PNPs entrap drug into nanosized carriers and release the drug at the target site (Desgouilles et al. 2003). PNPs are generally prepared by two main strategies – first, through dispersion of preformed polymers (top-down), and second, through polymerization of monomers (bottom-up) (Vauthier and Bouchemal 2009). PNPs can be termed a nanocapsules or nanosphere depending on the composition. Nanocapsule has a vesicular structure with a liquid core containing either oil or water surrounded by a solid shell. Nanosphere is a solid mass with polymer chains organized in matricial manner. The drugs can be entrapped in, dissolved or dispersed within, and adsorbed on PNPs (Couvreur et al. 1995; Guterres et al. 2007).

The techniques used for the preparation of PNPs from preformed polymers are solvent evaporation, salting out, nanoprecipitation, dialysis, and supercritical fluid technology involving the rapid expansion of a supercritical solution or rapid expansion of a supercritical solution into liquid solvent. On the other hand, various polymerization techniques utilized for the preparation of PNPs from monomer units are emulsion polymerization, microemulsion polymerization, miniemulsion polymerization, interfacial polymerization, and controlled/living radical polymerization (Rao and Geckeler 2011). The polymers used to design nanopharmaceuticals must fulfill certain requirements such as they should be nontoxic, nonimmunogenic, and biodegradable. The polymers should not accumulate in the body and hence should be eliminated within a span of time so that neither the polymers nor its degraded products accumulate in the body. Only a limited number of synthetic polymers (poly(lactide), poly(isobutylcyanoacrylate), poly(lactide-co-glycolide), poly(epsilon-caprolactone)) and natural polymers (chitosan, alginate, gelatin, albumin) are used as constituent of PNPs for drug delivery. Among these only a few are sanctioned for parenteral administration by healthcare specialists and others for oral or topical formulations and food industries (Vauthier and Bouchemal 2009).

Dispersion of Preformed Polymers

Several methods which are developed to synthesize PNPs by dispersing preformed polymers are discussed below.

Solvent Evaporation Method

Solvent evaporation method is an easy and very popular method which encapsulates lipophilic compounds efficiently. In this method, preformed polymers are dispersed in an organic phase and emulsified in aqueous phase. This is followed by homogenization by ultrasonication and solvent evaporation leaving the NPs behind as shown in Fig. 4.1 (Desgouilles et al. 2003). Two main strategies are used for the emulsion such as oil-in-water emulsion and water-in-oil emulsion. Surfactants are added to stabilize the nanoparticles (NPs). Several additives such as surfactants used in the synthesis process are removed by washing and then lyophilized. Polymers such as poly(organophosphazene), poly(d,l-lactic acid-co-glycolic acid), (monomethoxypoly(ethylene oxide)-poly(lactic acid)), etc. and stabilizers such as

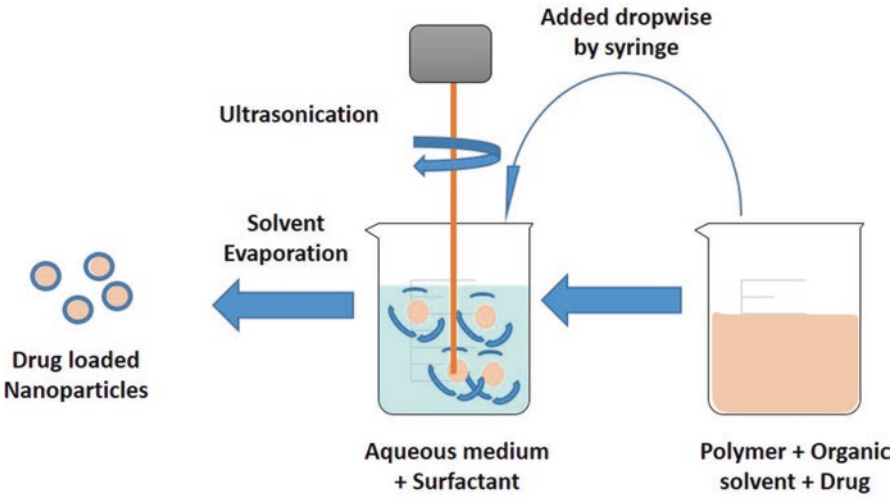

Fig. 4.1 Drug-loaded nanoparticle formation by solvent evaporation method. (Adapted from Ray et al. 2015)

poloxamine 908, PVA, Span 40, sodium cholate, sucrose, SDS, and Pluronic F-108 are used for the preparation of PNPs by this method (Rao and Geckeler 2011). This process is advantageous as it requires mild conditions such as ambient temperature and constant stirring. Process parameters such as power and duration of energy application influence size of the particles and drug content. Concentration of polymer and drug in organic phase, solvent volume, aqueous phase volume, surfactant concentration, molecular weight, and end groups of polymer are also among such factors influencing nanopharmaceutical properties (Hoa et al. 2012).

Salting Out Method

In salting out method, the use of organic solvent is replaced by water miscible solvent such as acetone in which polymers and drug are mixed. The solution is further emulsified into an aqueous solution mixed with salting out reagents and a colloidal stabilizer. Examples of few salting out reagents used are calcium chloride, magnesium chloride, magnesium acetate, sucrose, etc. Polyvinylpyrrolidone or hydroxyethylcellulose is used as colloidal stabilizer. Sufficient amount of water is added to the emulsion which induces formation of nanospheres efficiently, encapsulating the drug as shown in Fig. 4.2. The salting out reagents and the solvent are removed by cross filtration. This method is advantageous for proteins and heat-sensitive drugs as no heat is involved in this method. However, this technique is disadvantageous for lipophilic drugs (Nagavarma et al. 2012).

Nanoprecipitation Method

Nanoprecipitation method, otherwise known as solvent displacement method, uses three components: polymer, polymer solvent and polymer nonsolvent. Water

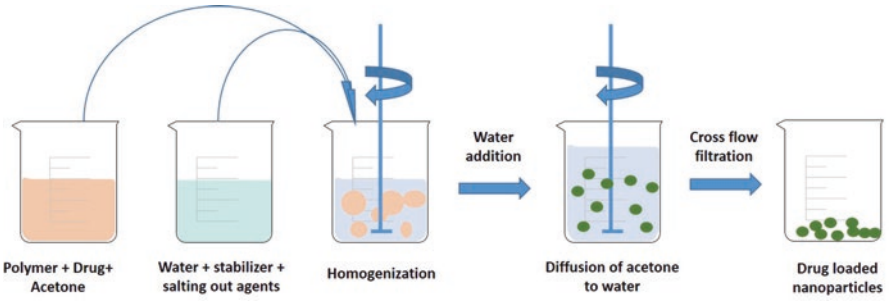

Fig. 4.2 Illustration of salting out method. (Adapted from Fonseca et al. 2013)

miscible organic solvent of immediate polarity is used to dissolve the polymer. Natural, synthetic, or semisynthetic polymers can be used for this method. The polymer solution is injected into an aqueous phase in presence or absence of surfactant. Polymer deposition occurs at the interface after displacement of semipolar water miscible solvent from the lipophilic solution (Nagavarma et al. 2012; Miladi et al. 2016). This method has three stages: nucleation, growth, and aggregation. Uniform particle size is obtained when the particles are separated out between nucleation and growth stage. This simple, fast, and reproducible method can generate both nanosphere and nanocapsule (Rao and Geckeler 2011).

Dialysis Method

In this method, polymer along with the drug is dissolved in an organic solvent miscible with water and dialyzed against water. Dialysis membrane used here is chosen with appropriate molecular cutoff. Dialysis results in displacement of solvent inside the membrane, and thus the polymer starts to aggregate due to loss of solubility. The nanosuspension thus obtained is freeze dried using a cryoprotectant to obtain a fine powder (Krishnamoorthy and Mahalingam 2015).

Supercritical Fluid Method

Supercritical fluid above their critical point exhibits properties of both liquid and gas. This method does not use organic solvent; therefore this is an environment friendly technology. Usually CO_2 is used as supercritical fluid because they are highly pure, odorless, colorless, safe, nontoxic, nonflammable, cost effective, and recyclable (Girotra et al. 2013). There are two most common processes of supercritical fluid technology: rapid expansion of supercritical solution (RESS) and rapid expansion of supercritical solution into liquid solvent (RESOLV). RESS involves dissolution of solute in a supercritical fluid followed by rapid expansion of the solution into ambient air through an orifice or capillary nozzle (Fig. 4.3a). The nozzle temperature is kept high. In this method, the products are microscale rather than nanoscale. RESOLV is an improved form of RESS technology in which supercritical fluid expands into a liquid solvent (Fig. 4.3b). The liquid here suppresses the growth of the particles, and hence nanosized products are obtained (Byrappa et al. 2008).

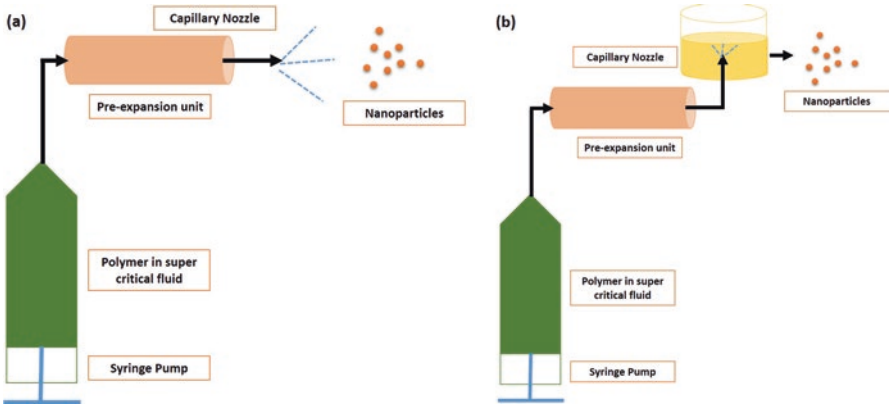

Fig. 4.3 Illustration of (**a**) rapid expansion of supercritical solution (RESS) and (**b**) rapid expansion of supercritical solution into liquid solvent (RESOLV). (Adapted from Rao and Geckeler 2011)

Polymerization of Monomers

PNPs can be designed for specific application and target using this approach. Techniques of monomer polymerization for the synthesis of PNPs are discussed below.

Emulsion Polymerization

Emulsion polymerization technique is a fast and scalable method. This method has been categorized into two groups based on utilization of organic and aqueous continuous phase. In organic continuous phase technology, monomers are dispersed into an emulsion or an inverse microemulsion or into a medium in which monomers are insoluble. However, this method has become less important as it uses toxic organic solvent, surfactant for protection of NPs from aggregation and initiators for polymerization. These components are finally eliminated from the product (Pinto Reis et al. 2006).

In aqueous continuous phase method, the monomers are usually dispersed in an aqueous phase without surfactant and emulsifiers. Here polymerization starts when monomer collides to initiator which can be a free radical or ion. Sometimes monomers themselves can be transformed into initiators when exposed to high-energy radiations (gamma-radiation, UV rays, and visible light). Initiated monomers or monomer radicals collide with other monomers and chain growth starts. During polymerization or after the termination of polymerization process, phase separates and solid particles are formed (Pinto Reis et al. 2006).

Miniemulsion Polymerization

This method is carried out in a shearing system comprising of a continuous phase, a dispersed phase, a surfactant, and an osmotic pressure agent. Miniemulsion process starts with the formation of small stable droplets of size 30–500 nm. These droplets polymerize without changing their uniqueness (Fig. 4.4) (Landfester 2003). The main difference between emulsion and miniemulsion polymerization lies in the

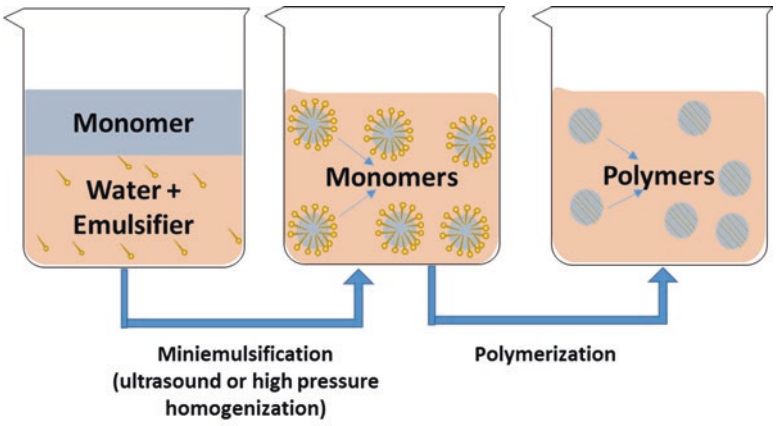

Fig. 4.4 Miniemulsion polymerization principle. (Adapted from Landfester 2003)

use of a low molecular mass compound (costabilizer) and high-energy sheer pressure (e.g., ultrasound). Costabilizer should be insoluble in aqueous medium and soluble in monomer droplets to prevent their diffusion. In this case, increase in costabilizer concentration results in free energy increase which again balances the interfacial energy to limit Ostwald ripening (Schork and Guo 2008). Miniemulsification allows functionalization of polymers by copolymerizing one or several functional monomers. Else polymers present in the dispersed phase of a miniemulsion are modified by degradation of polymer reaction or assembly of small molecules on polymers or grafting macromolecules (Crespy and Landfester 2010).

Microemulsion Polymerization

In microemulsion polymerization, smaller particle size with less average number of polymer chains per particle is obtained. The essential features of this technique are (1) polymerization proceeds under dynamic state; (2) during polymerization size and particle concentration; (3) chain transfer to monomer/exit of transferred monomeric radical/radical reentry events is operative; and (4) broad distribution of polymers (Capek 2001). Microemulsion is a thermodynamically stable transparent dispersion containing two immiscible liquids (oil and water) with surfactant. Polymerization involves free radical reaction with vinyl monomer in very fine oil droplets dispersed in water. Thermodynamic stability is lost after the formation of the first chain of the polymer. Very small amount of monomers can be solubilized within microemulsion droplet, whereas a large amount of surfactant and cosurfactant are required to stabilize the colloidal system. The reaction rate and the particle size vary with the type of surfactant used. Particle growth depends on the ratio of reaction rate to the transport of monomers between micelles (Al-Sabagh et al. 2012).

Interfacial Polymerization

Interfacial polymerization is an emulsification/solidification technique that generates nanocapsules (Mehrotra and Pandit 2015). This method involves dissolution of

two monomers in separate phases (continuous and dispersed phase) with reaction initiating at the interfaces of the two liquids. At the interface, cross-linking reactions like polyadditions and polycondensations or radical polymerizations occur which produce hollow particles. Oil-containing nanocapsules are formed at the interface of oil-in-water microemulsion. Interfacial polymerization of monomers results in water containing nanocapsules in water-in-oil microemulsion. Aprotic solvents promote nanocapsule formation, whereas protic solvents induce formation of nanospheres and nanocapsules (Rao and Geckeler 2011).

Controlled/Living Radical Polymerization

This technique is defined by a linear increase in the degree of polymerization with conversion and narrow molecular weight dispersion. A polymer with polydispersity index (PDI) of 2 can be termed as living agent. Living agent-mediated free radical polymerization generates three types of polymers: dormant chains, dead chains, and polymeric radicals. The former two chains can no longer be extended due to termination reactions. The later has negligible concentration, and its living behavior is determined by the ratio of the other two chains. This method has control over molecular mass and molar mass distribution. The macromolecular architecture, end functionalities, and impact on environment can also be manipulated which were lacking in radical polymerization technique.

Most of these methods are developed in an academic settings and lack scale-up facilities required for clinical use. Further, some techniques are only suitable for a specific type of nanomaterial and cannot be used for all types of nanomaterial design, thus requiring a unique synthesis and scale-up approach for a specific type of nanomaterial. Combination chemotherapy is widely used in clinics. However, nanoparticle-based delivery system for combination therapy is lacking due to the complex synthesis process required for multidrug nanoparticle system development (Anselmo et al. 2017). Another limitation of polymeric nanopharmaceutical synthesis is the toxicity caused due to the over use of detergent polyvinyl alcohol (Thakur et al. 2017).

Polymeric nanopharmaceuticals differ in size, structure, morphology, and drug release property based on the synthesis technique. Size of the polymeric nanopharmaceuticals is studied by electron microscopy (TEM, SEM, FESEM) and dynamic light scattering (DLS). Field emission scanning electron microscopy (FESEM) is carried out to study the morphology of the synthesized nanomaterials. Aggregation of nanoparticles will increase their size which is an important issue. Therefore, to study dispersity photon, correlation spectroscopy is utilized. Surface charge of the nanopharmaceuticals plays an important role in stability, and drug delivery is characterized by DLS. Surface chemistry, crystallinity, and the chemical structure of the nanomaterials are studied by XRD (X-ray diffraction), FTIR (Fourier transform infrared spectroscopy), and XPS (X-ray photoelectron spectroscopy). Drug entrapment efficiency and drug release properties are studied using UV-visible spectroscopy and HPLC. These techniques are listed in Table 4.2, and the detailed principle of these techniques has been described in Sect. 3. The particles with complex shape and geometrical size electron microscopy are used for the measurements which

Table 4.2 List of techniques employed to characterize polymeric nanopharmaceuticals

Polymeric Nanopharmaceuticals	Characterization	Techniques	References
Polymeric Nanocapsules loaded with anticancer drug (Imatinib-ITM)	Morphology	FESEM	Amgoth and Dharmapuri (2016)
	Cell viability	MTT assay	
	Cellular imaging	Laser scanning confocal microscopy	
	Drug release studies	Dialysis and UV-visible spectroscopy	
5-fluorouracil-loaded chitosan-coated polylactic-co-glycolic acid polycaprolactone nanoparticles	Particle size and poly dispersity index	Photon correlation spectroscopy	Badran et al. (2017)
	Zeta potential	Laser Doppler velocimetry	
	Entrapment efficiency	Separation of particles and estimation of nonentrapped fluorouracil by HPLC	
	Particle surface morphology	Scanning electron microscopy	
	Drug release studies	Dialysis and HPLC	
	Cell viability	MTT assay	
PLGA nanoparticle with an antiepileptic drug tiagabine hydrochloride	Compatibility studies between drug and polymer	Differential scanning calorimetry	Sakthivel and Arunachalam (2017)
	Particle size	Photon correlation spectroscopy	
	Surface charge determination	Zeta potential analysis	
	Drug entrapment efficiency	Spectrophotometric analysis	
	External morphological analysis	TEM	
	In vitro drug release study	Dialysis bag diffusion technique and spectrophotometric analysis	
Polymeric nanoparticles composed of N-isopropylacrylamide, methylmethacrylate, and acrylic Acid loaded with rapamycin	Size	Transmission electron microscopy	Bisht et al. (2008)
	Polydispersity	Dynamic light scattering	
	In vitro drug release	Separation and estimation of nonentrapped fluorouracil by centrifugation and spectrophotometry	
	In vivo pharmacokinetic studies	Drug release in blood studied by HPLC	
Lignin polymeric nanoparticles	Size	DLS, SEM	Azimvet al. (2018)
	Chemical structure	FTIR	
	Thermal stability	TGA-DTG	

require samples to be in dry state. 3D particle shape and size analyzer must be leveraged to analyze particles with complex physical features. Moreover, characterization of nanoparticles having dynamic physical properties is also a challenge (Anselmo et al. 2017).

Polymeric Nanomicelle Synthesis

Block copolymers comprising both hydrophilic and lipophilic parts have drawn attention for drug delivery. These copolymers including surfactants and amphiphilic monomers self-aggregate above their critical micellar concentrations in aqueous systems forming a core–shell structure with a hydrophobic core. The critical micellar concentrations of copolymers are lower than surfactants and therefore mostly preferred (Chen et al. 2016; Mitra et al. 2017). Polymeric nanomicelles can be prepared by direct dissolution, solution casting, and dialysis methods. In direct dissolution method, low molecular weight block copolymers with short length of insoluble block are used. Thermal, stirring, or ultrasound treatments are used to facilitate dissolution. In solvent casting method, the copolymer and drug are dissolved in organic solvent such as ethanol, and then the solvent is evaporated under vacuum. This results in the formation of a thin film which is then rehydrated with water for drug encapsulation (Cholkar et al. 2016). The direct dissolution method and solvent casting method are unsuitable when the core forming block is long and more hydrophobic. In such cases the polymers tend to solubilize large amount of poorly water-soluble drugs. Dialysis method can be used in these cases. In dialysis method, the drug and copolymers are dissolved in organic phase and dialyzed against water. It is suitable for water-insoluble copolymers. This process is although highly effective but time-consuming process. These limitations can be overcome by freeze-drying method (Mourya et al. 2011). It is a simple method which involves freeze-drying of freeze-dryable organic solvent containing copolymer, drug, and water resulting in freeze-thawed cake. This freeze-thawed cake is rehydrated, and nanomicelles are prepared (Puoci 2016). The size of the polymeric micelles depends on the preparation process, molecular weight of the amphiphilic block copolymer, aggregation number of the amphiphiles, relative proportion of hydrophilic and hydrophobic chains, and the quantity of solvent trapped inside the micellar core (Kulthe et al. 2012). Synthesized polymeric nanomicelles are characterized for their physical properties, cytotoxicity, and degradability by the techniques listed in Table 4.3. Polymeric micelle stability in physiological relevant media is an important aspect which needs to be evaluated. They lack stability in blood. Questions related to correlation between drug release and micelle stability are still unanswered. If the drug is hydrophobic and the core of the micelle is hydrophobic, then how the drug will be released? Whether enhancing drug load in polymeric micelle will have negative impact on micelle stability. Further the effect of freeze-drying and reconstitution on micelle stability is questioned. There are also limited of polymers which can be used for micelle synthesis. Though novel copolymers are reported for polymer micelle drug delivery systems, little attention is devoted for their cytotoxicity and

Table 4.3 List of techniques employed to characterize polymeric nanomicelle

Polymeric micelle	Characterization	Techniques	References
Hydroxyapatite hollow nanoparticles	Phases of the nanoparticles	XRD	Ye et al. (2010)
	Morphology	SEM, TEM	
	Chemical structure	FTIR	
	Estimation of drug loaded	TG measurement	
	Pore size	Nitrogen sorption	
Polymeric micelle	Size	DLS, static light scattering, atomic force microscopy (AFM), and TEM	Makhmalzade and Chavoshy (2018)
Radiolabeled polymeric nanomicelle	Structure	MALDI-TOF, UV spectroscopy, and FTIR	Oda et al. (2017)
	Critical micelle concentration	Spectrofluorometry, using pyrene P.A. as fluorescent probe	
	Size	DLS and SAXS	
	Surface charge	Zeta potential	
	Radioactivity	Gamma counter	
	In vitro stability	Thin layer chromatography (TLC)	
PEGylated polymeric nanomicelle	Composition and number-average molecular weight of copolymers	^{1}H-NMR	Lin et al. (2010)
	Molecular weight distribution	Gel permeation chromatography	
	Critical micellar concentration	Fluorescence spectrophotometer using pyrene as a fluorescence probe	
	Biocompatibility	Cell viability assay (MTT)	

biocompatibility properties. There are also limited methods for scaled-up synthesis of polymeric micelle-based nanopharmaceuticals (Lu and Park 2013; Owen et al. 2012).

Dendrimer Synthesis

Dendrimers are well-defined homogenous, branched, monodispersed nanosized radially symmetrical molecules (Abbasi et al. 2014). They consist of three major parts: a core, an inner shell, and an outer shell. There are numerous dendrimer synthetic strategies which can be categorized into divergent approaches and convergent approaches (Baig et al. 2015). Dendrimers are functionalized with drug molecules, gene, peptides, antibodies, MRI contrasting agents like dyes, etc. for medical

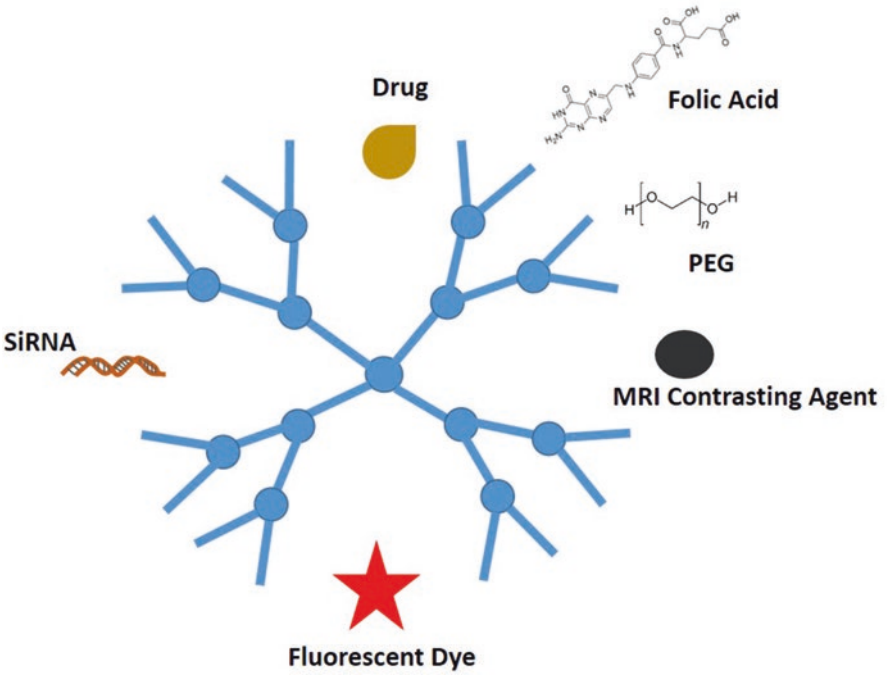

Fig. 4.5 Dendrimers as nanopharmaceutical. (Adapted from Madaan et al. 2014)

applications as shown in Fig. 4.5 (Madaan et al. 2014). Covalent attachment of PEG to the exterior of dendrimers reduces their toxicity and increases their permeability into tumor cells. Folic acid is conjugated to the dendrimers so that they can be targeted to the cancer cells due to the presence of folic acid receptors. Conjugation of folic acid is performed by EDC coupling. Doxorubicin in water is added dropwise to folic acid and PEG conjugated dendrimer solution. After stirring for 24 h, doxorubicin is loaded to dendrimers (Li et al. 2017). Dendrimers are characterized by spectroscopy, spectrometry, microscopy, and electrical and scattering techniques which are listed in Table 4.4.

Divergent Synthesis

It starts with addition of four arms (molecules) to the nitrogen of a multifunctional core molecule ethylenediamine through Michael addition reaction. Then in the second step, ethylenediamine is added so that they can react with the four arms of the molecule formed from the first step by amidation reaction. Excess ethylenediamine is used to avoid structural defects. This step is repeated multiple times to generate different generations of dendrimers. In this approach the yield is high with less purity (Gupta and Nayak 2015). A depiction of divergent method of dendrimer synthesis has been shown in Fig. 4.6a.

Table 4.4 List of techniques employed to characterize dendrimers

Dendrimers	Characterization	Techniques	References
Dendrimers	To monitor the synthesis of dendrimers	Ultraviolet-visible spectroscopy	Baig et al. (2015) and Caminade et al. (2005)
	Analysis of chemical transformation of end groups	NMR	
	Chemical alteration analysis on the surface of dendrimers	Infrared spectroscopy	
	Position changed π-π stacking interaction between end groups of modified PAMAM	Near infrared spectroscopy	
	Defect quantification during dendrimers synthesis.	Fluorescence	
	Characterization of small dendrimers (mass < 3000)	Mass spectroscopy	
	Precise determination of size, structure, shape, and chemical composition	XRD	
	Average radius of gyration (Rg) determination in solution.	Small-angle X-ray scattering (SAXS)	
	End group location determination	Small-angle neutron scattering (SANS)	
	Determination of hydrodynamic radius of dendrimers	Laser light scattering (LLS)	
	Imaging	SEM, TEM	
	Determination of substitution effectiveness of PANAM dendrimer surface	Electron paramagnetic resonance (EPR)	
	Electroactive end group interaction	Electrochemistry	
	Homogeneity and purity assessment of water-soluble dendrimers	Electrophoresis	
	Determination of glass transition temperature that depends on polymer composition, entanglement, and molecular weight	Differential scanning calorimetry (DSC)	
	Molecular dynamic processes (α-, β)	Dielectric spectroscopy (DS)	
	Chemical composition of dendrimers	X-ray photoelectron spectroscopy (XPS)	
	Dipole moment measurement for lactosylated PAMAM dendrimer	Sedimentation	
	Quantitation of NH_2 end groups of PAMAM dendrimers	Titrimetry	

(continued)

Table 4.4 (continued)

Dendrimers	Characterization	Techniques	References
Polyurethane dendritic wedges	Purity	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS)	Puapaiboon and Taylor (1999)
Poly(amidoamine) Dendrimers and their complexes with Cu^{2+}	Molecular weight, structure, and polydispersity	MALDI-TOF	Zhou et al. (2001)

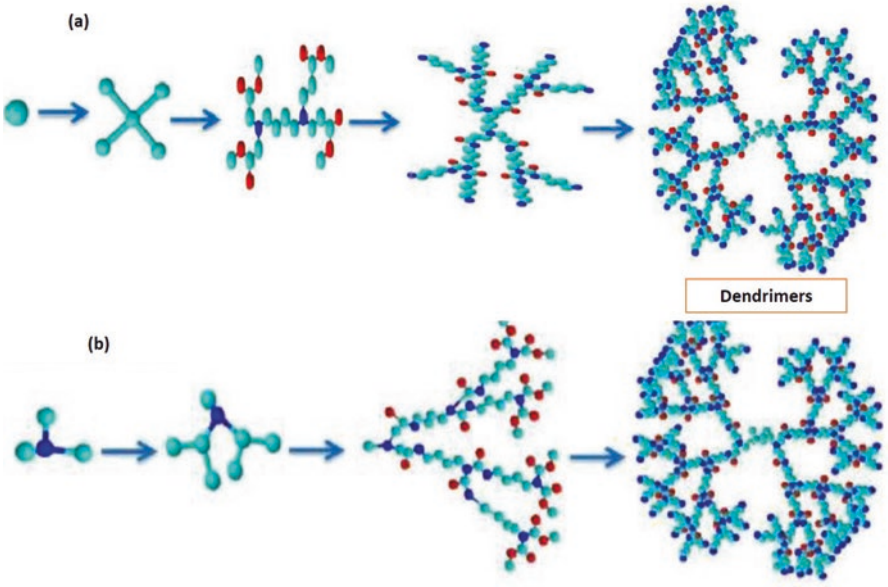

Fig. 4.6 Dendrimer synthesis: (**a**) divergent method and (**b**) convergent method. (Source: Baig et al. 2015)

Convergent Synthesis

The purity and structural defect issue is addressed in this approach. Uniformity and symmetry of the dendrimers synthesized are maintained, but the yield is very low. In this approach, the outermost arm of the final dendrimer is synthesized first and finally linked to a core molecule (Fig. 4.6b). Therefore final generation number is predetermined. However, larger size dendrimer creates steric hindrance during attachment to the core. This limitation is not observed in divergent synthesis technique (Abbasi et al. 2014; Gupta and Nayak 2015).

Dendrimers have been implemented in various practical applications. Still these are not propelled out of the academic curiosities to market for being extreme costly and their tedious procedures of synthesis (Hecht 2003). Moreover, limited drug loading capacity and inability to be surface modified are challenges for dendrimer-based nanopharmaceuticals (Pearson et al. 2012).

Lipid-Based Nanopharmaceuticals

Lipid-based nanomaterials are biocompatible and biodegradable and have ability to self-assemble to form membranes. They can form vesicles with volumes within and between the cells. Thus, they are explored as vehicle for molecular drug, biopharmaceutical agents (DNA, RNA interference effectors), and imaging agents

(magnetic resonance contrast reagents, radiometals, fluorescent probes) (Turánek et al. 2015). The three most important forms of lipid-based nanocarriers are (a) phospholipid-polymer nanomicelles, (b) lipid bilayer vesicular nanostructures (liposomes), and (c) solid lipid NPs (Fig. 4.7). Self-aggregation of monomers to form nanomicelles and liposomes is a bottom-up approach (Buse and El-Aneed 2010). However reducing the size of liposomes to nanoliposomes using energy input is a top-down approach. Nanomicelle and liposomes are made up of diglycerides, and the solid lipid NPs are made up of triglycerides. Preparation techniques of nanomicelle have already been discussed in the Sect. 2.2.

Nanoliposomes Synthesis

Nanoliposomes are 50–100 nm size vesicles filled with aqueous phase and surrounded by lipid bilayer membrane. Nanoliposomes enable efficient entrapment of water-soluble and insoluble drugs (Fig. 4.8). Double-emulsion method was reported

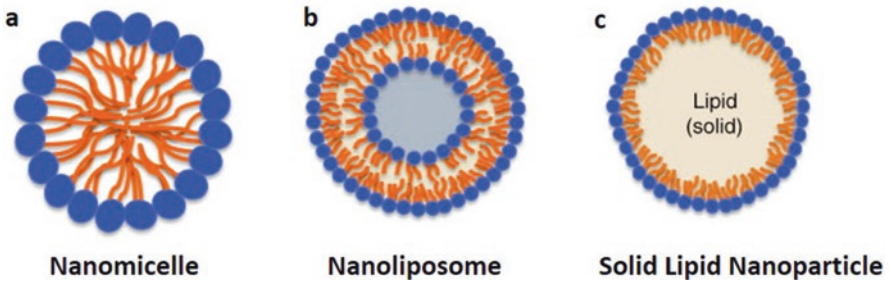

Fig. 4.7 Types of lipid-based nanocarriers. (Adapted from Wang et al. 2014)

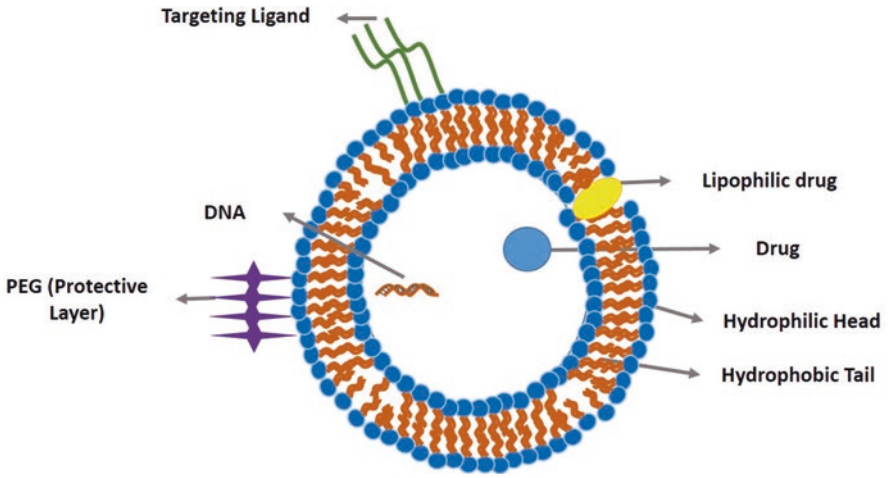

Fig. 4.8 Nanoliposomes as drug delivery vehicle. (Adapted from Malam et al. 2009)

to be the most efficient method for encapsulation of hydrophilic drug. Nanoliposomes have higher bioavailability and prolonged drugs residence time in the blood which makes slow release of drug release (Yang et al. 2013). Preparation of nanoliposomes requires phospholipids dispersed in water with sufficient amount of energy input. Energy input can be provided in the form of sonication, homogenization, heating, etc. so that the phospholipids arrange themselves into bilayer vesicles and a thermodynamic equilibrium is achieved in aqueous phase. Sonication technique uses titanium tipped probe sonicator to reduce the size of liposomes into nanoliposomes. Nanoliposomes are also prepared by extrusion method in which micrometric vesicles are extruded physically under pressure through a polycarbonate filter of defined pore size. Pharmaceutical industries use microfluidization technique to produce nanoliposomes encapsulating drugs. This technique is carried out in a microfluidizer in which high pressure is applied that induces cavitation and reduction in liposome size. The key advantages of this technique are large-scale production of nanoliposomes with reproducibility and control over particle size, and solutes to be encapsulated are not exposed to harsh conditions such as sonication and organic solvents. Heating method of nanoliposomes is used for gene transfer and drug delivery. In this method, prehydrated lipids and glycerol are dissolved after heating them at a temperature above their melting point under inert condition with constant stirring. Nanoliposome suspension is then allowed to anneal and stabilize under inert environment above their melting temperature for 1 h. Drugs can be added when the temperature drops or at the time of heating. An improvised heating method, known as Mozafari method, does not require prehydration of the liposomal ingredients (Mozafari 2010). Nanoliposomes have superior biocompatibility due to similarity with biological membrane. However, liposomes are less stable and can release drug at nonspecific sites. These challenges can be overcome by liposome modification and functionalization (Amoabediny et al. 2018).

Solid Lipid Nanoparticle Synthesis

Solid lipid NPs (SLNPs) are spherical colloidal carriers of 50–1000 nm average diameter. They contain solid lipid core which is stabilized by surfactant solution. Lipophilic drugs are solubilized in the solid lipid core. The important features of SLNPs are their small size and large surface area and superior drug loading ability. The interaction of phases at the interface makes them potent for pharmaceutical applications. SLNPs are composed of lipids which are solid at body temperature. They are synthesized by high-pressure homogenization (hot and cold homogenization), solvent emulsification, evaporation method, microemulsion-based method, etc. (Mitra et al. 2017). Preparation of SLNPs by homogenization technique is a top-down approach (Buse and El-Aneed 2010). These methods are summarized below.

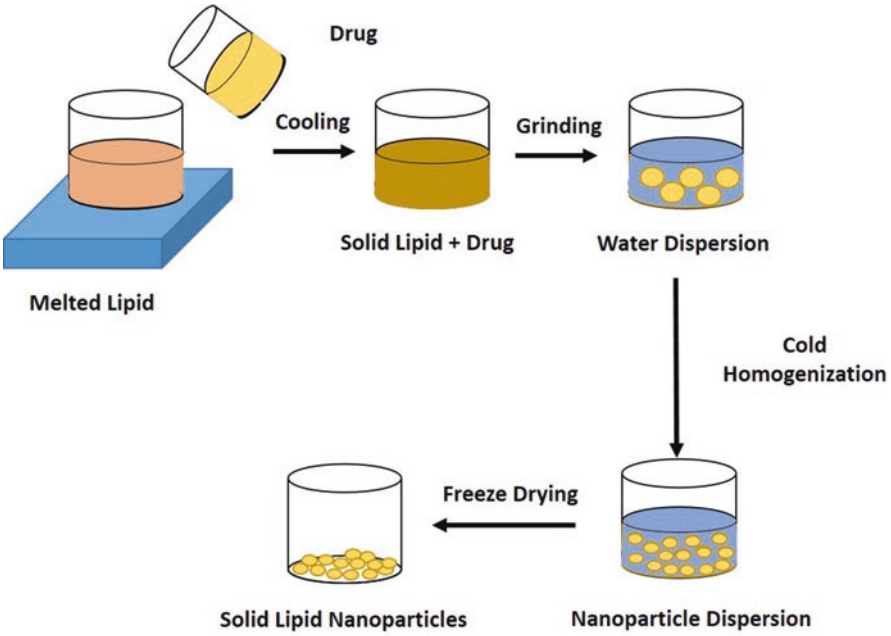

Fig. 4.9 Solid lipid nanoparticle synthesis by cold homogenization technique. (Adapted from Bondì et al. 2012)

Hot Homogenization Technique

Hot homogenization technique involves melting of solid lipids at a temperature above their melting temperature. To the melted lipid, drug is dissolved and dispersed. Melted lipid with drug (the above) is then dispersed into aqueous surfactant solution kept at a temperature similar to the melted lipid. Homogenization of this preemulsion results into nanoemulsion, and to start recrystallization of the lipids, its temperature is cooled down to room temperature. SLNPs are formed by recrystallization of the lipids through lyophilization (Fig. 4.9). This method is not suitable for temperature-sensitive drugs (Naseri et al. 2015).

Cold Homogenization Technique

Starting steps of hot homogenization technique are similar to cold homogenization technique. To the melted lipid, drug is added, and using dry ice or liquid nitrogen, temperature is lowered so that drug is distributed homogenously in lipid matrix. Microparticles obtained after pulverization of the solid lipid obtained by ball or mortar milling are dispersed in a chilled aqueous surfactant solution. This presuspension is homogenized to obtain SLNPs at room temperature. Preparation of SLNPs by cold homogenization technique is presented in Fig. 4.10. Here comparatively broader and larger particles are obtained than hot homogenization technique. This technique resolves some of the issues associated with hot homogenization technique such as drug loss to the aqueous phase during homogenization due to

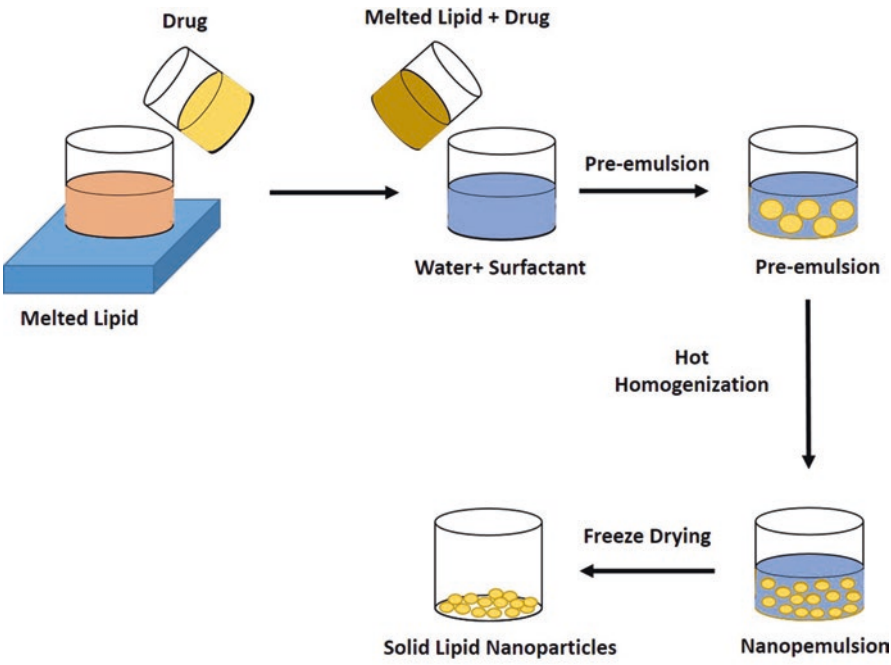

Fig. 4.10 Solid lipid nanoparticle synthesis by hot homogenization technique. (Adapted from Bondì et al. 2012)

partitioning, drug degradation due to high temperature, and uncertain polymorphic transitions of lipid in the crystallization step of the nanoemulsion (Yadav et al. 2014).

Ultrasonication or High Speed Homogenization
Here, the SLNPs are synthesized by ultrasonication or stirring at very high speed. There are few disadvantages of this method such as particle distribution at micrometer range, particle growth upon storage, and metal contamination during ultrasonication. Therefore usually this method is employed combined with high temperature (Mukherjee et al. 2009).

Microemulsification
In microemulsification method, a transparent mixture comprising of surfactant, cosurfactants, and a low-melting lipid dissolved in aqueous phase is stirred at 65–70 °C temperature. SLNPs are obtained when this microemulsion was added to cold aqueous phase under stirring condition. Typically the ratio of hot emulsion to cold aqueous phase ranges at 1:25 to 1:50 (Mukherjee et al. 2009).

Solvent Emulsification–Evaporation and Solvent Evaporation–Diffusion
Solvent emulsification–evaporation involves dissolution of lipids in an organic solvent followed by dispersion into an aqueous phase. Evaporation of this preemulsion under reduced pressure produces SLNPs (Mitra et al. 2017). In solvent evaporation–diffusion technique, a mixture of organic solvent partially miscible with water

saturated with water and lipid as prepared at elevated temperature. To this surfactant solution is added followed by surplus water addition. As a result organic solvent diffuses to aqueous phase and SLNPs precipitates (Mitra et al. 2017).

Homogeneous entrapment of drug is obtained by hot and cold homogenization process. Shell-enriched drugs result from phase separation of lipids and aqueous phase during SLNPs formation. During synthesis, if drug precipitates first, then core of the NP is usually filled with drug (Mitra et al. 2017). Several characterization techniques of lipid-based nanopharmaceuticals are listed in Table 4.5. Though SLNPs offer several benefits, there are some limitations too. There is a chance of degradation of the active components (DNA, peptides, and siRNA) during homogenization process. Similarly inactivation of the drug due to the heat generated during synthesis process is also an issue. Drug expulsion from the lipid matrix, coexistence of different colloidal forms, particle size, and shape variation are some challenges associated solid lipid nanoparticles (Kammari et al. 2017).

Carbon-Based Nanopharmaceuticals

Carbon-based NPs have useful physiochemical properties for which they have wide spectrum of biomedical applications such as therapeutics, sensing, and imaging. These NPs are fullerenes, carbon dots, and carbon nanotubes possessing unique electrical and tensile properties. Alternatively, carbon-based NPs can be classified as zero-dimensional, one-dimensional, and two-dimensional carbon NPs. Examples of zero-dimensional carbon NPs are fullerenes and carbon dots. Carbon nanotubes and graphene are considered as one-dimensional and two-dimensional carbon NPs, respectively (Singh et al. 2013; Liu et al. 2017).

Fullerenes

Fullerenes can be used in diagnostic purposes with radioactive metal enclosed inside them for magnetic resonance imaging and radioactive tracers. Fullerenes or buckyballs can also be used for drug transport, host immune response stimulator, antibody production, free radical scavengers, and photosensitizers (Sushen et al. 2017). Sixty carbon atoms are arranged in a soccer ball shape with 20 hexagons and 12 pentagons and 7 Å diameter in fullerenes. Various types of fullerenes include endo- and exohedral fullerenes, heterofullerenes, endohedral metallofullerenes, and alkali-doped fullerenes. Endohedral fullerenes consist of an atom enclosed inside the fullerene, whereas the atom is metallic in case of endohedral metallofullerenes. When alkali metal atoms are present between the fullerenes contributing valence electrons to the neighboring fullerenes, they are known as alkali-doped fullerenes. These are synthesized from larger fullerenes (C_{82} and C_{84}) as smaller fullerenes (C_{60}) are difficult to synthesize. Exohedral fullerenes or functionalized fullerenes are synthesized due to chemical reaction of fullerenes with other chemical groups.

Table 4.5 List of techniques employed to characterize lipid-based nanopharmaceuticals

Lipid-based nanopharmaceuticals	Characterization	Techniques	References
SLNPs and nanostructured lipid carrier	Shape and surface morphology	TEM, SEM, AFM, phase contrast optical microscopy, freeze fracture microscopy	Ganesan and Narayanasamy (2017)
	Size and size distribution	Optical microscopy, SEM/TEM, photon correlation spectroscopy (PCS)	
	Surface pH and surface potential	pH-sensitive probes, zeta potential measurement	
	Surface charge and electrophoretic mobility	Laser light scattering technique	
	Surface hydrophobicity	Two-phase partition, contact angle measurement probe, X-ray photoelectron spectroscopy, hydrophobic interaction, chromatography radiolabel, synchrotron radiation X-ray (SAX)	
	Rheology	Viscometer	
	Density	Gas pycnometer	
	Molecular weight	Gel permeation chromatography (GPC)	
	In vitro release	Dialysis membrane dissolution test apparatus	
Lipid nanoparticles containing hydrophobic metal nanoparticles	Morphology	Cryotransmission electron microscopy	Kulkarni et al. (2017)
	Estimation of total lipid concentration	Phospholipids C assay	
	Estimation of iron oxide concentration	Colorimetric thiocyanate assay	
	Particle size	DLS	
	In vivo pharmacokinetic and biodistribution studies	Measuring LNP labeled with ^{3}H – Cholesteryl hexadecyl ether using a scintillation counter	
	MRI contrast enhancement studies	In vivo magnetic resonance imaging	

Heterofullerenes contain nitrogen or boron atoms replacing one or more carbon atoms (Thakral and Mehta 2006; Bhatia 2016).

Synthesis of Fullerenes

Fullerene synthesis methods are of five types such as evaporation of high purity carbon by using resistive heating, arc discharge method using AC or DC, laser ablation of a rotating carbon disk in a furnace under flowing argon, flame production of soot from controlled combustion of benzene, and high-frequency inductive heating. These techniques produce carbon soots from which fullerenes are extracted using different solvents (Kroto et al. 1993). Resistive heating of graphite rods is performed in a helium atmosphere at 1300 °C and 1 kilobar pressure. This is the very first method of fullerene synthesis. However, the yield was less than 1% and required purification (Mojica et al. 2013). Carbon arc discharge method involves vaporization of graphite electrodes in low-pressure helium atmosphere by passing electric current through the electrodes generating an arc which produces soot-containing fullerene (Kyesmen et al. 2015). Fullerenes of C_{60} and C_{70} were obtained by combustion of benzene in a flat flame. Fullerenes can be produced in large scale through this technique. The fullerene concentration depends on the height above the burner surface (Mitra et al. 1992). Fullerene synthesis by laser evaporation of carbon starts with vaporization of a carbon source by laser beam. The vapor is carried away by inert gas stream through a zone where fullerenes are formed and grow. In this zone, the temperature is maintained between 1000° to 2000 °C. Fullerene is collected from this zone (Smalley 1994). Thermal evaporation using high-frequency oven at 2700 °C temperature yielded good amount of C_{60} fullerene. The soots obtained through the above techniques are solubilized in toluene. Fullerenes are separated using various chromatography techniques (Singh and Srivastava 1995). Shi et al. (2014) developed a tumoral pH-sensitive doxorubicin-loaded polyethyleneamine-derivatized fullerene to facilitate chemotherapy and photodynamic therapy. Doxorubicin was covalently conjugated with C_{60}-polyethyleneamine fullerene by pH-sensitive hydrazine linkage.

Carbon Dots

Carbon dots are biocompatible and low-cost materials having diameter below 10 nm with excellent fluorescent properties and high resistance to photobleaching. Due to these properties, carbon dots are applied in drug delivery, biological sensing, and imaging and environmental applications (Smagulova et al. 2017; Tuerhong et al. 2017). Carbon dots are of three types, graphene quantum dots, carbon nanodots, and polymer dots. Graphene nanodots are made up of less than five layers of carbon hexatomic ring honeycomb lamella with oxygen-containing functional groups which can be used for further modifications. Carbon nanodots are spherical and can be carbon NPs, without having a crystal lattice or carbon quantum dots with a crystal lattice. Linear polymers or monomers aggregate or cross-linked to form polymer

dot. Polymer chains attached to a carbon core can reassemble to form carbon dot (Zhu et al. 2015; Tuerhong et al. 2017).

Carbon Dot Synthesis

Carbon dots are synthesized from carbon resources such as graphite powder, carbon rods, carbon black, carbon nanotubes, carbon fibers, and candle soot by using an oxidizing acid (HNO_3, HNO_3/H_2SO_4 mixture). The surface is modified by oxygen-based groups to obtain graphene quantum dots, carbon quantum dots, and carbon nanodots (Zhu et al. 2015).

Graphene quantum dots are prepared by top-down and bottom-up approaches. Bottom-up approach includes solution chemistry and carbonization (Kellici et al. 2017). Top-down approach of graphene quantum dot preparation involves exfoliation of graphite powder by mineral acids and oxidizing agents in the first step also known as Hummer's method. In the second step, graphene oxide obtained from the first step is chemically reduced into graphene quantum dots (Paulo et al. 2016). Other top-down approaches of include electrochemistry, metal graphite intercalation, hydrothermal or solvothermal or special oxidation, laser ablation, arc discharge, and nanolithography by reactive ion etching (Zhu et al. 2015). Graphene quantum dots are produced by constant current electrolysis of graphite rod either in ethanol containing sodium methoxide or water as electrolyte (Muthurasu et al. 2016). Electrochemical exfoliation of carbon fibers in ionic liquid also formed graphene quantum dots (Yan et al. 2017). Electrolysis of graphite rod in a solution of tetrabutylammonium, DMSO, and refluxing with nitric acid and sulfuric acid generates multicolor fluorescent graphene quantum dots (Yuan et al. 2015).

Hydrothermal/solvothermal method of graphene oxide synthesis is the most used one among all the methods. In hydrothermal method, water is used as green solvent. Thermal treatment to graphene oxide sheets produces graphene sheets which were further cut by mixed acids under mild sonication. Graphene quantum dots were obtained when the suspension was transferred to a Teflon lined autoclave. In solvothermal reactions, organic acids are used as solvent to split graphene oxide to graphene quantum dot powder. Microwave hydrothermal method integrates hydrothermal method with microwave field, thereby accelerating reaction kinetics, shortening reaction time, and forming novel phases (Xu et al. 2013). Mass scale graphene quantum dots are also produced from graphene oxide by photo-Fenton reaction in which graphene oxide sheets reacted with Fenton's reagent under UV illumination (Zhou et al. 2012). Etching process is a well-known process of nanofabrication. Dry etching process involves breakage of a reactive gas in an etching chamber using a controlled radio frequency voltage. A plasma is created which disrupts the gas molecules into reactive fragments which when collide with the surface form volatile reaction products that etches the sample. If ions are the energetic species, then the etching process is known as reactive ion etching (Karmakar 2015). High-throughput patterning method fabricates uniform graphene quantum dots from graphene films using an etch mask (Fan et al. 2013).

Carbon nanodots are prepared by several existing methods such as laser ablation, pyrolysis, ultrasound, wet oxidation, microwave-assisted synthesis, hydrothermal

synthesis, and electrochemical etching (Wang and Hu 2014; Roy et al. 2015). Microwave-assisted carbon dot synthesis is popular among these methods for its low cost and one-step method. However, surface passivation is required to achieve luminescent carbon dots (Jelinek 2017).

Carbon nanodots and polymer dots can be produced in large-scale techniques involving dehydration and carbonization. These processes can be carried out by various approaches such as hydrothermal, microwave, combustion, pyrolysis in concentrated acid, carbonization in microreactor, microwave hydrothermal, and plasma-hydrothermal methods. These processes result in carbon dots with polydispersity as there is no control in the formation process. When precursors such as polycyclic hydrocarbons were used, size and molecular weight of graphene quantum dots were accurately controlled (Wang et al. 2014).

Quantum yield and photoluminescence of the carbon dots were enhanced by passivation and functionality which involves modification of reactive groups with other chemical groups. Photoluminescence of the carbon dots can also be tuned by surface or edge modification. Cross-linking enhances the photoluminescence property of nonconjugated polymer dots which is known as crosslink-enhanced emission effect (Zhu et al. 2014, 2015). Yao et al. (2016) constructed magnetic-carbon-quantum-dots-probe-labeled apoferritin nanocages for bioimaging and targeted therapy. They started with the synthesis of gadolinium-doped carbon dots by one-step microwave-assisted pyrolysis method. The carbon dot surface was coated with polyethyleneamine. Doxorubicin was loaded onto apoferritin cavity subsequently. Polyethyleneamine-coated and gadolinium-doped carbon dots were terminated with amine groups which were used for linking apoferritin loaded with doxorubicin by modified EDC-NHS method. The resultant compound was linked to folic acid.

Carbon Nanotube

Carbon nanotubes are cylindrical in structure with diameter in nanometer range and length in micrometers. These are single molecules made up of hexagonal network of covalently bonded carbon atoms seems as a graphite sheet being rolled up. The carbon tubes can be single-walled or multiwalled, where the former consists of a single layer of graphene sheet and the later consists of multiple layers of graphene sheets. They also have thermal conductivity, high tensile strength, high resilience, and high current density with changing electronic properties from metallic to semimetallic. In biomedicine, carbon nanotube has wide applications such as sensing, imaging, and drug and gene delivery (Singh et al. 2009).

Carbon Nanotube Synthesis

There are several methods of carbon nanotube synthesis among which three commonly used methods are carbon arc discharge method, laser ablation technique, and chemical vapor deposition technique. Laser ablation and arc discharge method use high temperature which is now replaced by chemical vapor deposition technique which uses low temperature. Additionally, the chemical vapor deposition technique

also has control over diameter, carbon nanotube length, alignment, density, orientation, and purity (Eatemadi et al. 2014). Arc discharge method involves generation of an arc between two graphite rods kept few millimeters distance apart inside an inert environment (Hornbostel et al. 2006; Sharma et al. 2015). It is a fast and cost-effective method and can be of two types depending on the use of catalyst precursors. In the absence of catalyst precursors, arc discharge method synthesizes multiwalled carbon nanotubes. Conversely, in the presence of catalysts, precursor and a complex anode comprising of graphite and a metal which can be Gd, Ni, Co, Pt, Fe, Ag, Pd, etc. or mixtures of Fe, Co, and Ni with other metals such as Ni-Y, Co-Pt, Co-Ru, Ni-Cu, Fe-Ni, Co-Ni, Co-Cu, Ni-Ti, Fe-No, Ni-Y, etc. synthesize single-walled carbon nanotubes. Higher yield of single-walled carbon nanotubes were obtained with anode comprising Ni-Y-graphite but with little control over chirality or alignment (Eatemadi et al. 2014).

A high-power laser is used to vaporize carbon from graphite block at high temperature in laser ablation technique. Both single and multiwalled carbon nanotube can be synthesized using this technique (https://sites.google.com/site/nanomodern/Home/CNT/syncnt/laser-ablation). The diameter of carbon nanotubes is controlled by laser power. High-power laser results in thinner carbon nanotubes. Metal particles are also added as catalysts to the graphite target for the synthesis of single-walled carbon nanotube. Other parameters such as target material (structure and composition), laser properties, buffer gas properties (flow and pressure), chamber conditions (composition and pressure), ambient temperature, and distance between the target and substrate material control the quality and quantity of the synthesized carbon nanotubes. This method results in low metal impurities as metallic atoms tend to evaporate from one end of the tube after its closure (Eatemadi et al. 2014). However, this method has two disadvantages: first, evaporation of carbon source makes it difficult for scale-up production with industrial standard, and second, production of mixed carbon nanotubes requires purification (Kaushik and Majumder 2015).

Chemical vapor deposition (CVD) method is one of the standard methods of carbon nanotubes synthesis. Both single- and multiwalled carbon nanotubes are synthesized by this technique with yield higher than laser ablation and arc discharge methods (Karimi et al. 2015). There are several types of chemical vapor deposition methods used to synthesize carbon nanotubes such as catalytic chemical vapor deposition (CCVD) which can be thermal CCVD or plasma-enhanced CVD, water-assisted CVD, microwave plasma CVD, radio frequency CVD, and hot filament CVD. CCVD is the standard technique for carbon nanotube synthesis (Pantapasis et al. 2017). Thermal CCVD involves pyrolysis of hydrocarbons such as acetylene, ethylene, propylene, methane, benzene, toluene, etc. or other carbon source such as polymers and carbon monoxide mixed with an inert gas. This mixture of gas flows inside a furnace containing substrates coated with catalysts (Ni, Fe, Co) with the furnace temperature maintained between 500 and 1200 °C. The carbon nanotube grows in various forms such as straight or coiled, thin or thick films, powder, aligned or entangled, or in a customized form on a predesigned substrate. The growth of carbon nanotube can also be controlled by temperature, atmosphere, carbon source,

and catalyst. Low temperature, i.e., 600–900 °C, yields multiwalled carbon nanotube, whereas higher temperature such as 900–1200 °C yields single-walled carbon nanotubes. This process has been scaled up to synthesize multiwalled carbon nanotube for commercial use. In plasma-enhanced CVD process, high temperature is replaced by plasma energy sources, and the hydrocarbon feedstocks used are in higher transition state to that of the transition metal catalysts. Multiwalled carbon nanotubes and nanofibers are synthesized in low temperature, whereas single-walled carbon nanotubes still require higher furnace temperature (Koziol et al. 2010). The characterization techniques employed for carbon-based nanopharmaceuticals are listed in Table 4.6.

Carbon nanomaterials have attracted a great deal of interest in the scientific community for their unprecedented physical and chemical properties. However, due to lack of large-scale fabrication process and difficulty in controlling important parameters, the development of carbon-based nanopharmaceuticals is still in progress. Manipulation of carbon nanotube limits their potential in pharmaceutical industries. Extensive exploration of carbon nanomaterial by scientific community can address these limitations (Nasir et al. 2018).

4.2.2 Inorganic Nanopharmaceuticals

Inorganic NPs can act as efficient nanocarriers due to their small size and stability against enzymatic degradation. Their optical, electronic, and magnetic properties can be tuned by changing their crystal phase, shape, dimensions, composition, and surface properties. Due to their small size, they can be excreted out through urine and feces. However, for FDA approval, their complete clearance after certain time period is required. The inorganic NPs can be widely used as therapeutic and diagnostic purposes. These NPs include semiconductor NPs, plasmonic NPs, magnetic NPs, silica-based NPs, and upconversion NPs. Inorganic NPs are synthesized both by bottom-up and top-down approaches.

Top-down approaches of inorganic NP synthesis include mechanical milling, mechanochemical processing, electroexplosion, sputtering, laser ablation, and nanolithography. Bottom-up approaches include chemical vapor deposition, chemical vapor condensation, plasma arcing, wet chemical method, hydrothermal, solvothermal, reverse micelle method, sol–gel method, sonochemical method, and green synthesis (Kumar and Kumbhat 2016). In bottom-up approach, NP synthesis employs three main components: metal precursors, reducing agents, and stabilizing or capping agents. The formation of NP involves two stages of nucleation and consequent growth. The size of the NPs depends on these two steps. These processes depend on temperature, pH, precursor, reducing agents, and stabilizing agents (Tran and Le 2013).

Table 4.6 List of techniques employed to characterize carbon-based and inorganic nanopharmaceuticals

Nanomaterials	Characterization	Techniques	References
Nanodiamond loaded with anti-HIV drug	Particle size, dispersity, and morphology	TEM	Roy et al. (2018)
	Estimating degree of crystallinity	XRD	
	Surface modification	Raman spectroscopy	
	Biocompatibility	MTS assay	
Carbon-based one-dimensional structure	Size	DLS, SEM, TEM	Barzegar (2015)
	Surface topography	AFM	
	Chemical composition analysis of surface	XPS	
	Purity of the SWCNT, defects, charge transfer, chirality, and diameter	Raman spectroscopy	
	Crystal structure	XRD	
	Thermal stability	TGA	
	Electronic properties carbon nanorods	Field-effect transistor geometry	
	Electrocatalytic activity of nanorods	Cyclic voltammetry	
Gold and silver nanoparticle	Size	TEM, SEM	Alaqad and Saleh (2016), Christy and Umadevi (2012), Menon et al. (2017) and Kim et al. (2008)
	Surface plasmon resonance	Surface-enhanced Raman spectroscopy and UV-visible spectroscopy	
	Functional groups on nanoparticles	IR spectroscopy	
	Crystallite size	XRD	
Copper nanoparticles	Surface plasmon resonance	UV-visible spectroscopy	Camacho-Flores et al. (2015) and Saranya et al. (2014)
	Size	TEM, SAXS, DLS, FESEM	
	Optical properties	UV-visible spectroscopy	
	Crystallinity	XRD	
	Electrochemical characteristics	Cyclic voltammetry	

(contined)

Table 4.6 (continued)

Nanomaterials	Characterization	Techniques	References
ZnO nanoparticles	Size	SEM, TEM	Talam et al. (2012)
	Crystallite size and phase	XRD	
	Optical properties	UV-visible spectrophotometer	
	Quantum confinement	Photoluminescence	
Iron oxide nanoparticles	Size	DLS, SEM, TEM	Ali et al. (2016), Cheng et al. (2012) and Campos et al. (2015)
	Molecular weight	Mass spectrophotometer	
	Surface chemistry	FTIR	
	Crystallinity	XRD	
	Shape, structure, size, and size transportation	SAXS	
	Magnetization	Vibrating sample magnetometry	
	Surface area	Brunauer–Emmett–Teller (BET)	

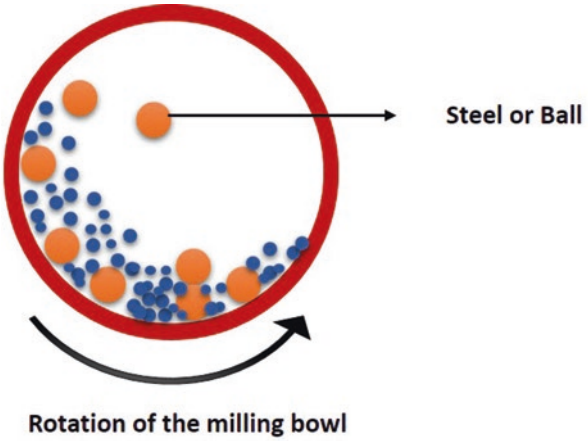

Fig. 4.11 Nanoparticle synthesis by mechanical milling process

Mechanical Milling

Mechanical milling process is widely used among all the top-down approaches to produce nanomaterials, nanograins, nanoalloy, nanocomposite, and nanoquasicrystalline materials. In this method, elemental or prealloyed powders are placed in a high-energy mill along with tungsten or carbide-coated balls (Fig. 4.11). The size of the particles is reduced due to the high-energy collision of these balls with the particles. The kinetics of the milling process depends on the energy transfer from the balls to the powder. Dense materials such as steel or tungsten carbide materials are preferred for the milling balls. Milling conditions (speed and duration, dry or wet milling, temperature), powder material, size, and size distribution of the milling balls govern the milling process (Prasad Yadav et al. 2012). Surfactants such as cationic, anionic, or charge neutral lubricants in the milling process control the nano-/microenvironment of the nanostructured materials so that NPs with improved dispersion are generated (Ullah et al. 2014).

Mechanochemical Processing

NP synthesis by mechanochemical processing involves repeated welding, deformation, and fracture of the reactant mixture. During milling process, nanometer-sized grains are regenerated, and chemical reactions occur at their interface. The chemical reactions that would generally require high temperature can take place at low temperature in a ball mill. Chemical reactions can occur in a steady-state manner or self-propagating combustive manner where the former results in a nanoscale mixture of products and the later results in micron sized particles. Addition of inert diluents to the starting powder reduces the reaction rate and hence avoids mechanochemical reaction. Selection of suitable chemical reaction path depends on stoichiometry of the starting material and milling conditions. Mechanochemical processing

can be used to synthesize NPs dispersed in a suitable matrix. The NPs are recovered by washing with appropriate solvents to remove matrix (McCormick et al. 2001; Tsuzuki and McCormick 2004).

Electroexplosion

Electroexplosion technique, an energy-efficient process is based on high-density current pulse passage through a metal wire which results in explosion of products and formation of NPs while passing through a gas atmosphere. This technique can be used to produce NPs of metals, alloys, metal oxides, and metal nitrides. Changes in NP characteristics are generated by changing the parameters of the process. Additionally, NPs obtained under normal conditions are stable but highly active in chemical reactions (Kryzhevich et al. 2017). Electrical explosion of Cu wire in n-hexane containing oleic acid generated surface oxide-free copper NPs (Lee et al. 2014) (Fig. 4.12). The Cu NPs produced through this technique were not aggregated.

Sputtering

Sputtering, a nonthermal process, involves physical ejection of clusters or surface atoms from a surface due to energetic bombarding of atomic or molecular species and momentum transfer between them. Sputter sources can be an ion gun or hollow cathode plasma or an electron gun. In case of magnetron sputtering, interaction is confined near the target region which is an advantage and not seen in diode and triode sputtering process (Kruis et al. 1998; Rajput 2015).

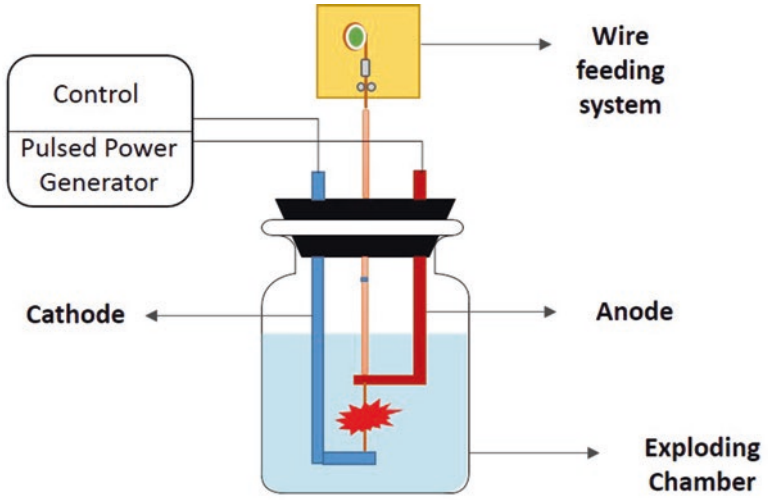

Fig. 4.12 Electroexplosion system. (Source: Lee et al. 2014)

Laser Ablation Technique

High purity NPs or nanofilms can be synthesized by very powerful and versatile laser ablation technique. In this technique, target is bulk sized, and the lasers are either excimer, pulsed yttrium, aluminum garnet, or femtosecond lasers. High-melting point materials can be easily deposited by this technique. α-Fe NPs can also be synthesized by pulsed laser ablation of an iron wire and bulk iron target at atmospheric pressure. Nanoparticle size can be changed by optimizing the laser parameters or ambient gas pressure (Altavilla and Ciliberto 2016).

Nanolithography

Synthesis of NPs with controlled and defined geometries can be achieved by nanolithography (Selvakumar et al. 2014). This technique generates surface with nanosized features by depositing, etching, or writing. Lithography is of different types: optical or photolithography (using light), X-ray lithography (using X-ray), i-beam lithography (using ions), or e-beam lithography (using electrons). Photolithography is a commonly used lithography technique. In the first step, surface is coated with desired substrate and a thin polymer layer of a positive or negative photoresist. Further the surface is covered by a predesigned light blocking mask. After UV exposure, the uncured portions are removed by the developer, while the cured photoresist remains on the substrate as a protective coating. Finally photoresist is removed after substrate etching process. When positive resist is used, direct duplication of the mask is obtained, whereas the use of negative resist generates inverse of it. Several advancements in lithographic techniques have been developed such as dip pen lithography in which a small tip deposits on a positive printing mode. Lithography is used in bioMEMS devices due to their applications in patterning in organic and inorganic materials, hydrogels, membranes, and ion-selective electrodes (Daraio and Jin 2012).

Chemical Vapor Synthesis

In this method, vapor phase precursors are bought into a hot wall reactor under conditions favorable for particle nucleation in vapor phase. The precursors that can be gas, liquid, or solid at ambient conditions are converted to vapor phase before delivering to the reactor. This technique also allows formation of multicomponent NPs using multiple precursors and doped NPs (Swihart 2003).

Chemical Vapor Deposition (CVD) and Chemical Vapor Condensation (CVC)

Deposition of solid particles from vapor or gas phase occurs on a heated surface through chemical reaction. The reaction needs activation energy to proceed which is provided by either high temperature above 900 °C as in thermal chemical vapor deposition method or by plasma at temperatures between 300 and 700°C as in plasma CVD. In laser CVD, laser thermal energy heats an absorbing substrate and pyrolysis occurs. In photolaser CVD, photon induces chemical reaction, and deposition occurs at room temperature. CVC involves pyrolysis of metal organic precursor vapors in a reduced pressure. Doping of NPs can be acquired by supplying two types of metal precursors in a reactor (Rajput 2015).

Sol–Gel Method

This method involves two phases: solution and gelation. Sol is colloidal solution of solid particles, and gel is an interconnected network of solid particles forming a continuous entity usually in a liquid phase. These phases are conserved throughout the sol–gel technology due to the chemical reactions taking place during the gel evolutions. This can be manipulated by altering initial precursors, catalysts, degree of solvation, gelation conditions, and physical processing of the gel. This technique allows formation of solid materials through gelation solution (Owens et al. 2016).

Chemical Reduction Method

In this method, metal salts solubilized in aqueous solution are reduced to zerovalent metal atoms at early stage of nucleation. These nuclei collide with other metal ions, metal atoms, or clusters to generate irreversible "seed" of stable metal nuclei. The diameter of the seed depends on the reducing potential of various metal salts, reducing agents, and strength of metal–metal bond. Examples of reducing agents are ascorbic acid; alkali hydrides, namely, $NaBH_4$, $NaB(C_2H_5)_3H$ and $LiB(C_2H_5)_3H$; and citric acid and their salts. Stabilizing agents prevent the synthesized NPs from agglomeration in this process. The stabilization can be electrostatic and/or steric. Ions on the NP surface form electrical double layer cause coulombic repulsion between the particles. Organic molecules on the NP surface act as protective shield. Stabilizing agents used in this process include polymers, (e.g., polyvinylpyrrolidone), surfactants, and organic ligand (e.g., quaternary amines, thiols) (Richards and Bönnemann 2005).

Reverse Micelle Method

Microemulsions are thermodynamically stable dispersion of two partially miscible or immiscible solvents stabilized by surfactants. Water-in-oil microemulsion is a type of microemulsions formed when water is dispersed in hydrocarbon-based continuous phase. Aggregates generated by thermodynamically driven self-assembly of surfactants, also known as reverse or inverted micelle. Two separate microemulsions containing different reactants are prepared and mixed together for NP synthesis. In water-in-oil emulsion, nucleation starts at the edge of the micelle due to oversaturation of reactants in the water. Growth occurs at the nucleation point due to incoming of more reactants by intermicellar exchange (Eastoe et al. 2006).

Hydrothermal and Solvothermal Method

Hydrothermal/solvothermal method involves performing chemical reactions in solvents at temperature around their critical point by heating with autogenous pressure. Therefore, this process is performed in a sealed reactor known as autoclave lined with Teflon or alloy. Inside autoclave extra can or beaker or tube made up of Teflon, gold, silver, or platinum can be used so that autoclave body is protected from solvents that are highly corrosive in high temperature and pressure. The organic solvents commonly used in solvothermal processes include toluene, methanol, 1,4-butanediol, and amines. Solvothermal process using water as solvent is known as hydrothermal. Nucleation starts when the solvent becomes supersaturated. Nuclei grow sequentially or through carriage of units in the solution and their attachment to the surface. Then these units move on the surface and attach to the growth sites. Factors such as additives, precursors, reaction time, and filling factor (the ratio of reactor volume filled with solution to the total reactor volume) affect the nucleation and growth of the NPs in this process (Li et al. 2015).

Sonochemical Method

Among the various gas phase techniques, ultrasound-assisted synthesis is extensively used. Sonochemistry and ultrasonic spray pyrolysis are two types of ultrasound-assisted synthesis techniques. Sonochemical reduction of metal salts is a fast process and does not require any chemical reducing agent. Sonolysis of water generates hydrogen radicals which are responsible for the reduction process. Different types of metallic colloids can be synthesized depending on the time, concentration, ultrasonic frequency, and organic additives. Sonolysis of aqueous solution of metal salts in the presence of air results in the formation of metal oxide NPs. Similarly, metal chalcogenides and carbides are prepared by sonolysis of aqueous solution of metal salts and a chalcogen. Physical effects induced by ultrasound have also been

utilized to deposit NPs onto substrates. Ultrasound spray pyrolysis process starts with ultrasonic nebulization of precursor solution and formation of liquid droplets of micrometer size. A gas flow carries these droplets to heating zone where the solvent evaporates, droplets shrink, and solute precipitates after supersaturation due to heating (Tsai et al. 2004; Bang and Suslick 2010).

Electrospinning

A typical electrospinning setup comprises of a syringe pump to hold sample, a high-voltage source, and a collector. In this process, a polymer solution is kept in a syringe, and at the needle tip, the polymer solution is held by surface tension. High-voltage charge is induced within the polymer when electric field is applied resulting in charge repulsion within the solution. Eventually a jet is initiated due to the charge repulsion which overpowers the surface tension. As this jet travels, solvent is evaporated, and nanofibers are produced. A collector is placed to collect the nanofibers. Electrospinning is an important and popular technique in tissue engineering and drug delivery field (Pham et al. 2006; Sill and Recum 2008).

Green Synthesis of Nanoparticles

NP synthesis by physical and chemical methods is expensive and high-energy consuming and uses toxic and environmentally hazardous chemicals. These processes also generate several byproducts and waste materials which are equally toxic. Moreover, chemical method of NP synthesis increases reactivity and toxicity. Therefore, their use in biomedical field is sometimes questionable. Biogenic approach of NP synthesis is ecofriendly, sustainable, and economical, and the synthesized NPs are free of toxic chemicals (Hussain et al. 2016). Importantly, green synthesis explores biological entities to use their inherent biochemical processes to transform metallic ions to NPs (Baker et al. 2013). During the past decade, it has been established that a wide range of biological entities such as plants, algae, diatoms, bacteria, fungi, yeast, viruses, and single cells are used to synthesize NPs (Parveen et al. 2016). Each biological entity has varying processing capabilities and different mechanisms for the metal and metal oxide NP synthesis. Therefore, the choice of the biological entity is important for the synthesis of NP of desired characteristics (Shah et al. 2015).

Microbe-Mediated Nanoparticle Synthesis

Microorganisms, both unicellular and multicellular, are reported to synthesize NPs through bottom-up approach by reduction/oxidation of metal ions using biomolecules such as enzymes, sugars, and proteins secreted by them. NPs synthesized by microbes can be applied in various fields such as drug delivery and sensors for

diagnostics owing to their less toxicity. Metallic NPs, metal oxide NPs including magnetic and nonmagnetic oxide NPs, sulfide NPs, and other miscellaneous NPs are synthesized using microorganisms (Li et al. 2011).

Microbes synthesize inorganic materials either extra or intracellularly, where the former is desirable for easy recovery (Khandel and Shahi 2016). Usually metal ions are trapped on the surface or inside the microbial cells due to electrostatic interaction between ions and cell membranes. They are then reduced to NPs in the presence of enzymes such as NADH and NADH-dependent nitrate reductase enzymes. Some metallophillic bacteria have the genetic system which enables cellular detoxification against heavy metals such as cadmium, copper, nickel ions, etc. through reductive precipitation, complexation, and efflux mechanisms (Li et al. 2011). In case of extracellular nanoparticle synthesis, there are possibly two routes of NPs synthesis: (i) the microbes release biomolecules to the external medium which reduces the metal ions to form NPs. (ii) NPs are synthesized inside the microbe and then released to exterior (Singh et al. 2015).

Plant-Mediated Nanoparticle Synthesis

Biosynthesis of metallic NP using living plants (intracellular), plant extracts (extracellular), and phytochemicals are considered as an appropriate substitute to traditional physical and chemical methods (Iravani 2011). The use of agricultural wastes for NP synthesis further lowers the cost of NP synthesis many folds (Gan and Li 2012). The size of the plant-mediated synthesized NPs is comparable to those obtained from physical and chemical methods (Parsons et al. 2007). However, to control the size of the NPs, purified phytoconstituents such as polyphenols, proteins, and organic acids are used to synthesize NPs (Basha et al. 2010; Tamuly et al. 2014).

Plant Biomass-Mediated Nanoparticle Synthesis

Researchers employ the ability of plant species to accumulate heavy metals (phytoextraction) and detoxification (phytoremediation) to synthesize metallic NPs. Using *Medicago sativa* grown in a solid media, gold and silver NPs were synthesized. Using intracellular route, other plants are also reported which synthesize various NPs including *Brassica juncea*, *Festuca rubra*, *Triticum aestivum*, *Chilopsis linearis*, *Avena sativa*, and *Sesbania drummondii*. The intracellular route of NP synthesis has disadvantages such as deviation in reducing and stabilizing potential of the bioorganic compounds in different plant parts and conditions resulting in variations in degree of polydispersity and morphology of the nanoparticles. Additionally, the downstream processes such as recovery of the NP and their purification are tedious jobs. Due to these drawbacks, this approach has been outdated (Dauthal and Mukhopadhyay 2016).

Plant Extract-Mediated Nanoparticle Synthesis

This is an extracellular process in which the phytoconstituents are extracted from the plants and directly used for the NP synthesis. The protocol starts with collection

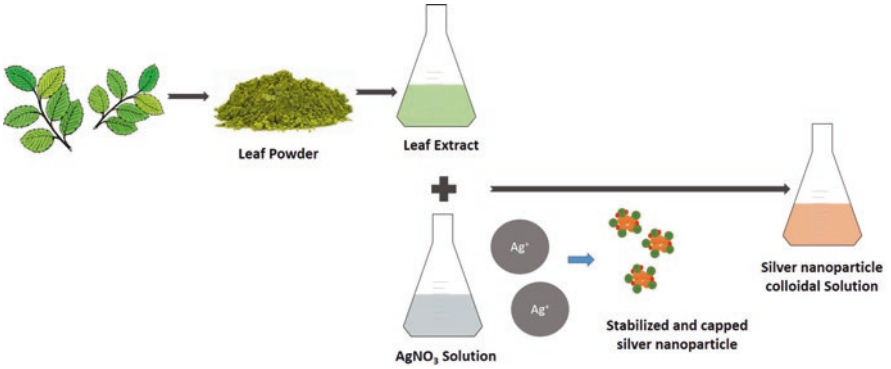

Fig. 4.13 Depiction of silver NPs synthesis using plant extract

of plant parts and cleaning to remove dust, epiphytes, and necrotic plants. The cleaned plant parts are dried under shade and ground to powder by domestic blender. Extract of the plant is prepared by hot or cold maceration process. The extract is filtered and mixed with metal ions such as silver nitrate or gold chloride solution at optimized concentration to synthesize NPs (Fig. 4.13). Synthesis of NPs is verified by UV-visible spectrum analysis. Plant extract containing amino acids, proteins, enzymes, polysaccharides, vitamins, and secondary metabolites (alkaloids, tannins, phenolics, saponins, terpenoids) act as both reducing and stabilizing agents in this technique (Ahmed et al. 2016). Biosynthesis of metallic NPs using plant extracts is simple, one-step method, fast, ecofriendly, and biocompatible method for the synthesis of NPs. This process has easy downstream processing and NP recovery and can be easily scaled up. Green synthesis method generates highly reactive nanoparticles (FCC structure) with energetically favorable shape. For various commercial applications, nanoparticles with spherical shape and growth along (111) plane are desirable for their reactivity (Dauthal and Mukhopadhyay 2016; Iravani 2011).

Inorganic nanoparticles are characterized by high-throughput standardized methods. Still there is a need of rapid assessment of their toxicity under different environment conditions. In vivo toxicity of inorganic nanoparticles cannot be predicted based on the results of in vitro toxicity assessment with cells and proteins. Further, preclinical studies using animal models mimicking human system with standardized and validated protocol should be developed so that the effect of nanopharmaceuticals can be explained for a given dose and duration of exposure. The biological interaction of the nanoparticles inside body during their life cycle along due to occupational exposure and from environmental release should be governed and regulated (Hofmann-Amtenbrink et al. 2015). Although nanoparticle-based imaging offers enhanced detection sensitivity and improved chemical and optical stability, drawbacks such as tissue damage on exposure to UV radiation used for quantum dot excitation prevent their extensive use in clinics. Magnetic susceptibility artifacts arising from T2 MRI contrast agents and toxicity of heavy metal-containing nanoparticles should be corrected in nanoparticle imaging system (Kim et al. 2018).

4.3 Characterization of Nanopharmaceuticals

Nanopharmaceuticals synthesized from above synthetic procedures exhibit different physical, chemical, and biological properties than their counterpart bulk materials due to their nanosize and large surface to volume ratio. The properties of the nanostructures change according to their size and shape. Various advanced techniques and sophisticated instruments have been developed by different interdisciplinary areas aiming to observe nanostructured objects with high resolution. These techniques are discussed below.

4.3.1 Imaging Through Electron Microscope

Electron microscopes were developed to overcome the limitations of simple optical microscopes which have a resolution power around 0.2 μm 200 nm. On the other hand, electron microscopes have a resolution power around picometer range. Electron microscopy and scanning probe microscopy are two types of electron microscopy imaging techniques. In case of electron microscopy, electron beam is focused on the sample to obtain an image, whereas in scanning probe microscopy, a probe of nanometer range scans over the sample surface revealing the 3D structure of the material surface. Further, there are two types of electron microscopes: scanning electron microscope and transmission electron microscope. A probe beam scans the sample surface in scanning electron microscope, whereas in transmission electron microscope, a probe beam passes through the sample which is differentially refracted and absorbed, and an image is created (Kumar and Kumbhat 2016).

Scanning Electron Microscopy (SEM)

SEM allows imaging of NPs, nanofibers, nanocoating fracture surface, and surface configuration of polymer composites with clarity. Atomic flat surfaces, multilayered nanowires, carbon nanotubes, and biomolecules are also characterized by SEM. In tissue engineering, cell growth and scaffold construction are visualized through SEM. The resolution of SEM is up to 5 nm. Moreover, elemental composition and crystallographic, magnetic, and electrical properties of the sample can be obtained through SEM with advanced instrumentation. For SEM, sample should be dry and coated with conducting material such as gold. The dried powdered sample is kept over a sample holder which is kept inside SEM instrument, and vacuum is created. A beam of electrons is focused onto the sample. Surface structure and elemental composition is analyzed from the backscattered electrons, secondary electrons, auger electrons, and X-rays generated from the surface. Elemental composition and their proportion are analyzed by SEM in conjunction with energy-dispersive X-rays (EDX). Samples should be dry and conductive for SEM analysis (Kumar and Kumbhat 2016; Linkov et al. 2013).

Transmission Electron Microscopy (TEM)

TEM involves imaging using transmitted electrons through the specimen. Image of high resolution, i.e., X1000000, with diffraction information is obtained from TEM. TEM also characterizes nanocomposites and reports about their extent of dispersion, intercalation, and exfoliation. High-resolution transmission electron microscopy (HRTEM) is another tool used for NP characterization with resolving power around 1 Å. Morphology (particle shape and size) and information on crystallography, elemental composition, phases (lattice spacing), topography, and elemental mapping of the nanomaterials are obtained through TEM analysis. Sample preparation for TEM is an extensive process for biological samples though nanopowders can be easily deposited onto a copper grid. Biological samples are unstable in vacuum; therefore they are fixed using fixating agents such as glutaraldehyde and paraformaldehyde, dehydrated and stabilized using resins. Biological samples and polymeric nanocomposites are cut into ultrathin slices under cryogenic condition so that they can be electron transparent. Mostly nanoclusters, nanocrystals, nanoparticles, quantum dots, nanowires, nanotubes, dendrimers, and biomolecules are analyzed using TEM. Image contrast can be enhanced using metal stains such as lead citrate (Kumar and Kumbhat 2016). Sample thickness limit for TEM is 200 nm. Sample preparation technique is time consuming, and ultrahigh vacuum is required for atomic scale resolution. For biological samples there is a chance of material damage by the electron beam (Linkov et al. 2013).

4.3.2 Scanning Probe Microscopy

This technique uses a physical probe that scans the sample surface by raster scanning motion, and an image is generated (https://physics.boisestate.edu/kimresearch/instruments/). This technique is useful for measurement of surface topography and surface properties at atomic scale (Bottomley 1998). Both conductive and nonconductive nanomaterials, nanotubes, and nanocrystals are characterized by scanning probe microscopy. Scanning tunnel microscopy and atomic force microscopy are the most widely used variants of scanning probe microscopy (Mozafari et al. 2005).

Scanning Tunnel Microscope (STM)

In this microscopy, the conducting sample surface and the metal tip are brought very close to each other. When the tip-sample distance is in the range of 1–2 nm, a tip-sample voltage drives a measurable tunneling current through vacuum. STM with spectroscopic capabilities allows atomic resolution imaging of conducting surfaces and manipulation of atoms and molecules of the sample (Natelson 2015). STM allows to study surface topology, surface structures, surface reactions, and single

point defects of the nanomaterials. The sample should be conductive, and electrical and mechanical noises should be minimized for STM (Linkov et al. 2013).

Atomic Force Microscopy (AFM)

Considering the limitations of STM, AFM was developed. Using AFM, visualization of biological entity-coated samples is possible in air, controlled environment even in liquid which is impossible for STM. This is a nondestructive technique with high 3D spatial resolution. Besides, it also provides information about size, morphology, surface area, surface texture, and volume distribution. In this technique, the tip is mounted on a cantilever which moves over the sample surface. Height changes caused by particles are converted to voltage change. Minute deflection occurring on the cantilever due to the force between cantilever and sample surface is detected optically. It is necessary for NPs to be fixed onto a sticky surface such as mica, TempFix, and Tacky Dot slides (Maver et al. 2016). Slow scanning speed of AFM that gives rise to thermal drift is a limitation of this technique. AFM images might get affected by hysteresis of the piezoelectric material (Linkov et al. 2013). Applications of AFM in nanopharmaceuticals characterizations are wide including drug discovery, drug delivery, and drug transport (Maver et al. 2016).

4.3.3 Spectroscopy Techniques

Spectroscopy deals with the interaction of matters with electromagnetic radiation. NPs have different optical properties according to their size, shape, agglomeration state, and refractive index near NP surface. They are characterized using absorption, transmission, and scattering of the electromagnetic radiations. A lot of information about nanomaterial properties can be decoded using different wavelength of electromagnetic radiations.

UV-Visible Spectroscopy

UV-visible spectroscopy uses light of UV-visible spectral region. When the light energy matches with the electronic transition within the molecule, it is absorbed to promote electrons to high-energy orbital. Optical spectrophotometer records the absorbance degree at each wavelength and presents a graph of absorbance versus wavelength (https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/Spectrpy/UV-Vis/spectrum.htm). The wavelength at which maximum absorbance is recorded is a characteristic for a specific NP. Metallic NPs such as gold and silver are characterized by plasmon resonance absorption which gives a characteristic color when present in solution. When these NPs are excited with electromagnetic radiation, collective oscillation of conduction band electrons results in wavelength-selective

absorption which is called as surface plasmon resonance (Krajczewski et al. 2017). If an incident light has an energy close to the excitation energy of surface plasmon oscillations, the local electromagnetic field near the particle can be higher in magnitude than the incident fields. The incident light around the resonant peak wavelength is scattered very strongly then. Particle morphology (size and shape), interparticle coupling (e.g., state of aggregation), and dielectric environment (surrounding medium and substrate coating) determine the exact position, shape and intensity of the localized NP surface plasmon resonance (Grigorchuk 2012).

Vibrational Spectroscopies: Fourier Transform Infrared Spectroscopy (FTIR) and Raman Spectroscopy

FTIR and Raman spectroscopies complement each other in investigating the structure of the molecule. Vibrations that are strong in infrared spectrum are weak in Raman spectrum and vice versa. The IR spectrum reveals the molecular structure and functional groups and bonds present in the sample qualitatively through their characteristic frequency of the bands. It covers 50 to 12,500 cm^{-1} region. When IR passes through an organic sample, some frequencies are absorbed, and others are transmitted according to the type of bond and functional groups present in them. However, FTIR is unable to provide quantitative data (Garhwal 2011).

In Raman spectroscopy, when sample is illuminated with monochromatic laser, light interaction of the sample with the light results in scattering of light. Much of the scattered light has frequency equal to the frequency of incident light and constitutes Rayleigh scattering. When the scattered light has frequency different from incident radiation, they are known as Raman scattering. Raman spectrum presents intensity versus wavelength shift. Raman spectrum is recorded over 10 to 4000 cm^{-1}. Using Raman spectroscopy, molecules are identified as vibrational information is specific to chemical bonds and symmetry (Bumbrah and Sharma 2016). Raman signals are weak for metals as their surface plasmons limit light penetration. Particles with fluorescence properties also mask the Raman spectra. Quantitative analysis of different phases is not possible in Raman spectroscopy (Gouadec and Colomban 2007).

X-Ray Photoelectron Spectroscopy (XPS)

XPS technique is applied to broad range of materials and provides quantitative and chemical state information from the surface of the materials (https://www.phi.com/surface-analysis-techniques/xps-esca.html). Nanolayers, nanoparticles, thin corrosion, block polymer, and copolymeric light-emitting diodes are characterized by XPS. In XPS, X-rays of well-defined energy are bombarded to the substrate so that they interact with the core electrons present around the nucleus of the different atoms present in the sample. If these electrons escape, they are emitted at a well-defined kinetic energy which is different for different types of atoms and orbitals and even the same type of atom and orbital in different binding states. An analyzer

separates electrons with different kinetic energy and represents a result in a spectrum. This spectrum shows the photoelectron intensity, types of atoms present in the surface layer, and their abundance. High vacuum is required for this method, and during analysis there is a chance of sample degradation. Errors arise in analyzing chemically heterogeneous surfaces through XPS (Claesson 2007).

4.3.4 Scattering Techniques

Dynamic Light Scattering (DLS)

DLS is mostly used to determine size and size distribution of the NPs in solution. It measures the hydrodynamic radius of the NPs. This technique relies on the Rayleigh scattering from the suspended NPs that undergo Brownian motion. The hydrodynamic diameter of the NPs is calculated from their diffusion speed on laser light illumination. DLS provides agglomeration status of the NPs in solution. Size of proteins, polymers, micelles, carbohydrates, and NPs can be characterized by DLS. However DLS has some limitations. This concept is applicable for monodisperse sample and unable to resolve polydisperse samples (Fissan et al. 2014).

X-Ray Diffraction (XRD)

Uniqueness of X-ray diffraction pattern for each crystallite is used for the identification of a chemical compound. Bulk polycrystalline solids, membranes, liquid-suspended particles, and biological objects are analyzed by XRD. In pharmaceutical industry, XRD is used in drug development, testing, and production. The consistency in production of drug is checked by XRD as a quality control process. Crystallite size, space between lattice planes, and crystalline phase are determined by XRD. It also helps in studying the nature of the polymers and nanocomposites. It allows differentiation between optical isomers, enantiomers, and cis/trans isomers. The entire electron density pattern can be mapped using XRD. During operation, when X-rays falls over a crystal or powder, they scatter producing a diffraction pattern which informs about the atomic arrangement in a crystal. XRD is a nondestructive technique with several other advantages such as easy sample preparation, high sensitivity, reliability, simplicity, depth profiling, and low maintenance cost. This technique is limited by peak broadening at small-sized crystal. Recent innovation and synchrotron radiation in XRD analyzes bulk polymers, biopolymers, and multilayered semiconductors with 0.5–500 nm thickness. The International Centre for Diffraction Data (ICDD) has stored powder diffraction data of more than 600,000 substances. Information on the structures of organic, inorganic, and organometallic substances are also stored in Crystallography Open Database. The Cambridge Structural Database contains structures of small organic and organometallic molecules (Chauhan and Chauhan 2014).

Small-Angle X-Ray Scattering (SAXS)

The ability to resolve features in the range between 1 and 100 nm at high resolution of SAXS technique is used to characterize the nanoparticle in real time and under realistic sample environment. Particles such as aerosols, micelles, minerals, and particles synthesized through sol–gel reactions are studied by SAXS. SAXS measures only the electron density differences. SAXS results should be compared with the TEM results. TEM analysis is limited to a particular analysis area, whereas SAXS provides structural data averaged over macroscopic sample volume. SAXS allows to determine particle dimensionality, size distribution, core–shell morphology edge length of polyhedral particles and nanoparticle concentration (Li et al. 2016; Allec et al. 2015).

Zeta Potential Analysis

Zeta potential of a drug affects its stability, intracellular trafficking, transfection efficiency, and sustainability in bloodstream. Surface charge of nanopharmaceuticals is an important aspect for drug delivery. Without surface modification, the NPs are opsonized and massively cleared from the bloodstream by macrophages of reticuloendothelial systems. Particle size and zeta potential of a gene carrying nanomaterial should be examined for successful transfection (Honary and Zahir 2013). In a colloidal system, zeta potential of a particle can be defined as the potential difference between the stationary fluids attached to the particle surface and the dispersion medium present away from the particle surface. Zeta potential indicates the stability of the colloidal system. High zeta potential indicates more dispersion of the particles, whereas less potential denotes less repulsion and more attraction between the particles resulting in agglomeration. In a zeta potential measuring instrument, the potential is calculated from the electrophoretic movement of the particles. When electric field is applied to a colloidal system, they move according to their surface charge which scatters the laser light. The electrophoretic mobility can be calculated from the phase change in the scattered light (Greenwood 2003; Kirby 2010; Hanaor et al. 2012).

4.3.5 Surface Hydrophobicity Studies

Surface hydrophobicity of nanopharmaceuticals is also one of the surface properties which decides their fate, transport, bioavailability, and toxicity. Surface hydrophobicity of engineered NPs are characterized by surface tension, surface adsorption of hydrophobic or hydrophilic molecules, and affinity coefficient. Surface tension is measured by contact angle measurement employing sessile drop Young–Laplace method. Surface adsorption method assesses the affinity of the nanoparticle to a standard hydrophilic or hydrophobic molecule. In the partitioning method, the

nanoparticles are distributed between two immiscible liquid phases such as water and octanol (Xiao and Wiesner 2012). Xiao and Weisner performed Rose Bengal dye and naphthalene adsorption, octanol–water affinity coefficient, and contact angle determination experiments to characterize surface hydrophobicity of carbon- and metal-based NPs with and without surface coatings.

4.3.6 Drug Release Studies

In vitro drug release from nanopharmaceuticals can be studied by direct diffusion method in which NPs carrying drug are dispersed in a buffer for a period of time. The drug released into the buffer is analyzed spectrophotometrically (Nimesh et al. 2006; Anitha et al. 2011; López Goerne et al. 2013). Bohrey et al. (2016) used dialysis bags to perform drug release experiment. Other methods of drug release studies include size-exclusion chromatography, ultrafiltration, and spectral changes between free and entrapped drug (Fugit 2014).

4.3.7 Biocompatibility Studies

It is proposed that NPs of size less than 10 nm act similar to a gas and can easily enter human tissues resulting in disruption of normal biochemical environment of cells (Bahadar et al. 2016). Therefore, nanopharmaceuticals should be characterized for its biocompatibility to ensure safe drug release with minimum toxicity. Studies regarding interaction of a drug with the target environment are also essential (Naahidi et al. 2013). NPs as biosensors and delivery vehicle of drug and gene come in direct contact with blood; therefore, their hemocompatibility is also studied. Hemocomapatibility studies are performed by evaluating red blood cell aggregation, hemolysis study, and coagulation behavior (Li et al. 2012). Cellular toxicity of the nanomaterials in various cell lines is evaluated by assays such as cellular morphology assay and cell viability assays. Cell viability assays are mostly dye-dependent assays which involve inclusion, exclusion, or conversion of an added dye. Enzymatic conversion of a dye precursor in living cells versus dead cells also indicates cell viability in these assays. Dye-dependent assays are quantified colorimetrically. These assays include formazan-based assays (MTT, MTS, WST assays), lactate dehydrogenase assay (LDH assay), mitochondrial membrane potential (MMP) assay, ATP-luciferin luminescence, adenylate kinase release, and thiobarbituric assay. Dyes such as resazurin, trypan blue, neutral red, and Coomassie blue are used for cell viability assays. Cell stress assays are performed to evaluate the nonlethal cell injuries and changes in cell behavior due to nanomaterial-induced changes in the surrounding environment. Altered reactive oxygen species levels, reduced glutathione (GSH) levels, cytokine protein expression, cell stress-related gene, and protein expression due to cellular stress are also measured. Particle uptake assay and

cell visualization distinguish normal cells from injured or dying cells (Jones and Grainger 2009). In vivo studies of NP toxicity in appropriate animal model are requisite due to the growing apprehensions of FDA (Bahadar et al. 2016; Adabi et al. 2017). Zebrafish embryos were used as a vertebrate model system, and their mortality was assessed after a period of NP exposure (Lathamuthiah et al. 2015).

4.4 Conclusion

The use of nanotechnology in pharmaceutical industry has revolutionized site-specific drug delivery, therapy, and disease diagnosis. Nanopharmaceuticals are synthesized and formulated to meet the desired pharmacokinetics and therapeutic value with minimized toxicity. Therefore, synthesis of nanopharmaceuticals of desired properties is an important step of nanopharmaceutical development. Researchers are continuously working on this part which is evident from the number of articles published in this area. Once synthesized, characterization of these nanopharmaceuticals is equally important. High-end sophisticated instruments are now being used to characterize their physical, chemical, and biological properties. Understanding the mechanism of nanopharmaceutical uptake, transport, interaction with biological components, and their removal from the biological system is essential. Toxicity assessment of these nanopharmaceuticals toward biological systems and environment is also required. In order to establish nanopharmaceuticals as novel therapeutics for patients, selection of appropriate synthetic approach, assessment of their therapeutic value, toxicity, and quality at each step during large-scale production are compulsory.

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Chapter 5
Electrospun Nanofibers as Carriers in Dermal Drug Delivery

Meryem Sedef Erdal and Sevgi Güngör

Contents

Abstract Nanotechnology has opened a new direction in biomedical sciences. Nanomedicine and nanodelivery systems offer multiple benefits in treatment of diseases by site-specific and target-oriented delivery of many drugs. Among the various forms of nanocarrier systems, nanofibers have recently proved to be a versatile carrier system for drug delivery applications due to their attractive properties such as target-specific, prolonged delivery of drugs, and ease of fabrication. Polymeric nanofibers can be produced using several techniques such as phase separation, self-assembly, and electrospinning. Electrospinning is a versatile, cost-effective, and scalable technique using electrostatic forces to produce fine fibers from polymer solutions or melts. The nanofiber production by electrospinning enables higher drug loading and entrapment efficiency compared to other nanodelivery systems prepared by other methods. Electrospun nanofibers can be fabricated from a wide variety of solutions of either natural or synthetic polymers, as well as combinations thereof. The type of the polymer can be chosen depending on the treatment, on the nature of the drug, and on the compatibility with the biological environment. During

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the last several decades, polymeric nanofibers have been explored as controlled drug delivery systems for dermal, transdermal, oral, oromucosal, parenteral, and ocular routes. Recently, electrospun nanofibers have gained more popularity for the topical and transdermal drug delivery and wound dressing applications. Especially, the unique architectural properties like nanoscale morphology, porous structure, and flexibility of electrospun nanofibers make them a suitable option for developing novel wound dressings. However, despite the numerous attractive features of nanofiber composites in drug delivery applications, there are certain major drawbacks which need to be overcome. Drug stability, initial burst release, and scale-up problems foremost require to be solved before bringing nanofiber technology into mainstream drug delivery technologies. In this chapter, first, the basic concepts of electrospinning process and the characterization techniques of electrospun nanofibers are discussed. Then, the most widely used polymers in the composition of drug-loaded nanofibers are presented, and recent applications of nanofibers in dermal drug delivery and wound healing are described.

Keywords Electrospinning · Nanofibers · Polymers · Topical drug delivery · Transdermal drug delivery · Wound healing

5.1 Introduction

Novel drug delivery systems are used to improve the efficiency and safety of active compounds by carrying them on the site of action at a specific rate (Vasita and Katti 2006; Akduman et al. 2016). Particularly, nanocarriers like liposomes, nanoparticles, and nanoemulsions have demonstrated increased drug absorption, penetration, half-life, bioavailability, and stability. Among the many forms of nanocarrier systems, nanofibers have recently proved to be a versatile carrier system for drug delivery applications due to their remarkable properties such as high drug-loading capacity, high encapsulation efficiency, target-specific, prolonged delivery of drugs, and ease of fabrication (Ravikumar et al. 2017).

Polymeric nanofibers are defined as solid fibers with a nanoscale diameter. They can be produced using several techniques such as phase separation, self-assembly, and electrospinning (Melanko et al. 2009). Among these techniques, electrospinning is the mostly preferred one because of its versatility for adaptation to both laboratory- and industrial-scale production processes (Akduman et al. 2016). Electrospun nanofibers can be fabricated from a wide variety of solutions of either natural or synthetic polymers, as well as combinations thereof (Zanin et al. 2011). Natural polymers offer the advantage of being very similar to the macromolecules present in the extracellular matrix (ECM). They are usually biodegradable and biocompatible. Synthetic polymers, on the other hand, have great flexibility in synthesis and modification. In most cases, it is preferable to fabricate composite nanofibers comprising both natural and synthetic polymers (Hu et al. 2014).

The unique properties of electrospun nanofibers such as high surface area to volume ratio and high porosity make them suitable carriers for both hydrophilic and lipophilic drugs, and research studies have been especially focused on topical wound healing and tissue engineering applications recently (Kyzioł et al. 2017). Concerning dermal drug delivery, electrospun nanofibers provide an opportunity for encapsulation of different active agents such as antifungal and antimicrobial therapeutics, antibiotics, proteins, and growth factors (Karthikeyan et al. 2015; Zhao et al. 2016). Drug release rate from nanofiber mats can be tailored by modulation of nanofiber morphology, porosity, and composition. Nanofiber mats can provide sustained drug release, which reduces the application frequency, and consequently increase the patient compliance to the treatment (Goyal et al. 2016; He et al. 2014).

In this chapter, first, the basic concepts of electrospinning process and the characterization techniques of electrospun nanofibers are discussed. Then, the most widely used polymers in the composition of drug-loaded nanofibers are presented, and recent applications of nanofibers in dermal drug delivery and wound healing are described.

5.2 Electrospinning of Nanofibers

Electrospinning is a versatile, cost-effective, and scalable technique using electrostatic forces to produce fine fibers from polymer solutions or melts. Electrospinning was patented firstly by Formhals in 1934 (Frenot and Chronakis 2003). It is a top-down nanomanufacturing process and usually conducted at room temperature with atmosphere conditions. A classical electrospinning setup, shown in Fig. 5.1, consists of three basic components: a syringe pump fitted with a metallic spinneret, a high voltage supply, and a conductive collector (Bhardwaj and Kundu 2010; Liao et al. 2008).

In the electrospinning process, a high voltage is applied to the polymer solution, and with the use of the syringe pump, the polymer solution is delivered to the tip of spinneret at a constant rate (Kamble et al. 2017; Zanin et al. 2011; Zamani et al. 2013). When the electrostatic charge is larger than the surface tension of the polymer droplet at the tip of the nozzle, the droplet elongates to form the "Taylor cone." Then, a fiber jet extrudes from the cone and moves toward the fiber collector (He et al. 2014; Seif et al. 2015). As the solvent evaporates during this process, a mat of ultrafine solid nanofibers is collected as a final product. The evaporation patterns determine the porosity of the nanofibers (Sharma et al. 2015; Frenot and Chronakis 2003).

The nanofiber production by electrospinning enables higher drug loading and entrapment efficiency compared to other nanodelivery systems prepared by other methods (Pelipenko et al. 2015). Among all the developed methods for drug loading into nanofibers, mixing the drug with a polymer solution prior to electrospinning remains the most predominant. Generally, lipophilic drugs are loaded in lipophilic polymers, while hydrophilic drugs are loaded in hydrophilic polymer solutions

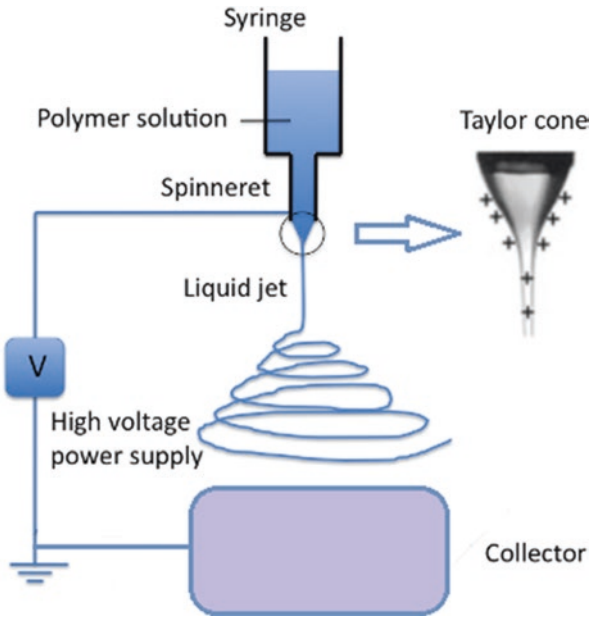

Fig. 5.1 Schematic representation of the electrospinning process

(Kamble et al. 2017; Zamani et al. 2013). For this purpose, the drug is dissolved or dispersed in the polymer solution to achieve encapsulated drug through a single-phase electrospinning procedure. A common problem in this technique is the loss of activity of the incorporated biological molecules (e.g., enzymes and growth factors) during the electrospinning process (Mickova et al. 2012). Therefore, several electrospinning techniques have been developed to obtain nanofibers with different shapes and structural characteristics (He et al. 2014).

Coaxial electrospinning is a modified version of electrospinning that enables production of drug-loaded fibers with core–shell morphology (Zamani et al. 2013) (Fig. 5.2). The coaxial configuration involves two capillaries which permit simultaneous electrospinning of two separate polymer solutions into core–shell structured nanofiber (Zanin et al. 2011). The shell polymer improves sustained and prolonged release of the active agent. It also prevents the direct contact of the drug with the external environment. Therefore, to encapsulate specific materials such as biological agents and growth factors, coaxial electrospinning is preferred (Manuel et al. 2016). Mickova et al. (2012) produced core–shell nanofibers with embedded liposomes. They have suggested that the liposomes remained intact during and after coaxial electrospinning in contrast with blend electrospinning. The main drawbacks of coaxial electrospinning are the need for a specialized electrospinning setup and the complication of controlling of multiple feed rates (Canbolat et al. 2013).

Emulsion electrospinning is another method for the encapsulation of the drugs into core–shell structured nanofibers. Briefly, this technique involves the emulsification of drug solution in the polymer solution and the electrospinning of the obtained emulsion. The application of emulsion electrospinning process has been resulted in

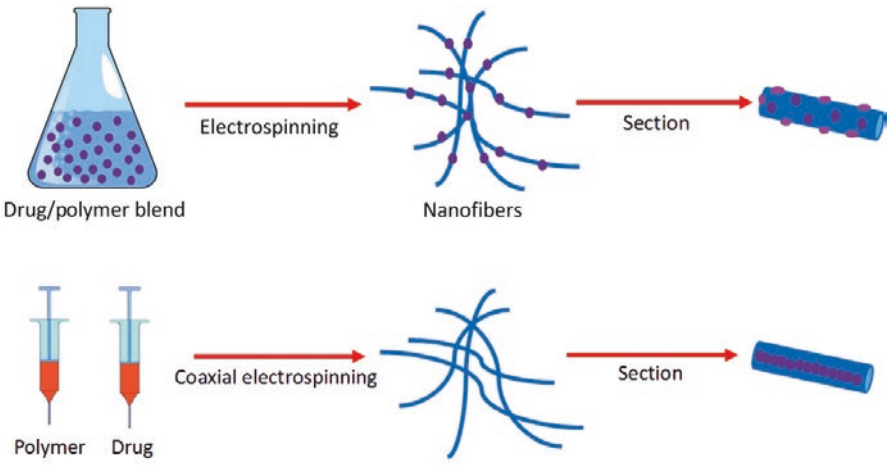

Fig. 5.2 The difference between the blend (conventional) and coaxial electrospinning processes

the controlled release of drugs and enhanced bioactivity of sensitive compounds (Nikmaram et al. 2017). The primary advantage of this technique is the absence of organic solvent usage (Manuel et al. 2016). On the other hand, emulsion electrospinning requires the inclusion of surfactants, which may generate toxicity to the biological systems (Zhang et al. 2018).

Other techniques to incorporate drugs into nanofibers could be listed as (a) physical adsorption by immersing electrospun nanofibers into drug solution wherein electrostatic interactions occur; (b) chemical method, which involves modifying the surface properties of nanofibers and covalent bounding of drug molecules to the surface of preformed nanofibers; and (c) bioconjugating drug molecules such as enzymes, DNA, or growth factors to nanofiber surfaces (Pelipenko et al. 2015; Sharma et al. 2015; Kamble et al. 2017).

It can be found in the literature that certain techniques such as phase separation and self-assembly have also been used to prepare drug-loaded polymeric nanofibers. However, the obvious disadvantages of these techniques can be listed as material limitation and time-consuming and complex processing (Table 5.1) (Morie et al. 2016; Zanin et al. 2011). Compared with other methodologies, electrospinning can be used for many formulations and is more reproducible when system and process parameters are controlled (Manuel et al. 2016).

Conventional electrospinning is accepted as an easy and efficient method; however, the big problem limiting its industrial applications is the electrospinning production rate versus commercial fiber production rate. Multineedle electrospinning and needleless electrospinning came to the forefront among the several methods which have been developed to increase the productivity of solution electrospinning process (Valipouri 2017; Zhao et al. 2016). Multineedle electrospinning technique is based on multiplication of the jets using multineedle constructions. Jets instability and mutual interference have been reported as the main limitations of this method

Table 5.1 Advantages and disadvantages of phase separation, self-assembly, and electrospinning techniques

Nanofiber production technique	Advantage	Disadvantage
Phase separation	Minimum equipment requirement Process can directly fabricate a nanofiber matrix Batch-to-batch consistency is achieved easily Mechanical properties of the matrix can be tailored by adjusting polymer concentration	Limited to specific polymers Laboratory-scale process Fiber dimensions cannot be controlled
Self-assembly	Process can fabricate smaller nanofibers	Complex and laboratory-scale process Fiber dimensions cannot be controlled
Electrospinning	Cost-effective Long, continuous nanofibers can be produced Scale-up possibility	Jet instability

(Dubský et al. 2012). The needleless electrospinning technology however created an opportunity for the scale-up the conventional electrospinning process to an industrial production level (Zhao et al. 2016). The setup which consists of a rotating drum immersed into a bath of a liquid polymer has been commercialized under the brand name Nanospider™. Nanospider™ technology is a patented, needle-free, high-voltage, free liquid surface electrospinning process and is able to fabricate polymeric nanofibers in diameter range of 50–300 nm into nonwovens (Petrik 2011; Kamble et al. 2017).

5.3 Factors Affecting Electrospinning

Although electrospinning is considered to be a simple production technique, a number of parameters can influence the formation and structure of the obtained fibers. The process parameters and the system parameters known to affect nanofiber properties are shown in Fig. 5.3. By appropriately adjusting all or some of these parameters, fibers with desired morphology and diameter can be obtained (Frenot and Chronakis 2003; Supaphol et al. 2012).

The physicochemical properties of the polymer solution have a significant impact on the final morphology and characteristics of the formed electrospun fibers (Morie et al. 2016; Bhardwaj and Kundu 2010). The polymer concentration influences both the viscosity and the surface tension of the solution. Within the optimal concentration range of the solutions, uniform fibers can be obtained (Supaphol et al. 2012). It was reported that the fiber diameter increases with an increase in the polymer solution concentration (Huang et al. 2003). A low viscosity favors an efficient stretching of the polymer jet and thus the formation of thinner fibers. However, the viscosity of the polymer solution must be high enough to prevent the jet from collapsing into

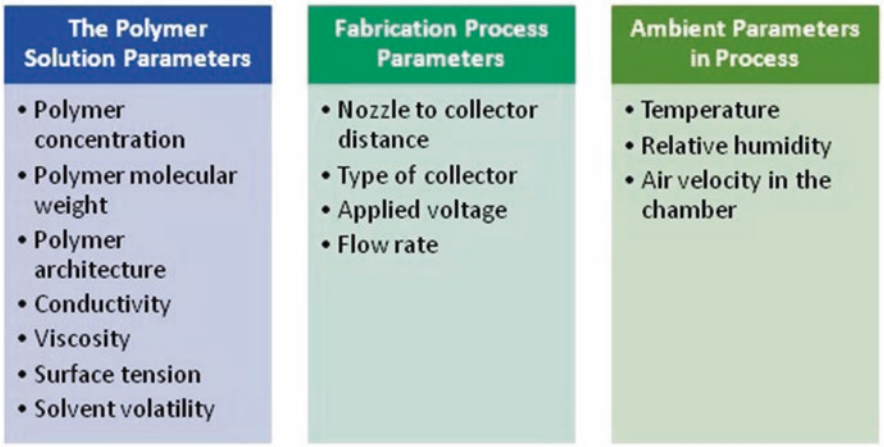

Fig. 5.3 Process parameters and system parameters affecting nanofiber production by electrospinning method

droplets before the solvent has evaporated (Frenot and Chronakis 2003; Gomes et al. 2015).

The vapor pressure of the solvent should be suitable so that it evaporates quickly enough for the fiber to maintain its integrity when it reaches the collector (Ramakrishna et al. 2005). Solvent volatility has been reported to affect the porosity of fibers (Morie et al. 2016). The conductivity of the polymer solution can be altered by adding ionic salts. A low conductivity leads to a decrease in stretching behavior of the jet and to thicker nanofibers (Gomes et al. 2015). It was suggested that the radius of the fiber jet is inversely related to the cube root of the solution conductivity (Supaphol et al. 2012). The surface tension of polymer solution is another significant parameter, and electrospinnability could be enhanced by reduction in the surface tension (Huang et al. 2003; Wali et al. 2018).

Fabrication process parameters can greatly affect fiber formation and structure. The applied voltage, polymer flow rate, nozzle to collector distance, and type of the collector can influence the formation of nanofibers with bead-like defects (Bhardwaj and Kundu 2010; He et al. 2014). The distance between the capillary and the fiber collector has an impact on fiber drying and should be kept at minimum. If the distance is too long, the electrostatic force cannot overcome the surface tension, and electrospraying instead of electrospinning occurs (Thakkar and Misra 2017). Polymer flow rate also has an impact on fiber size and can influence fiber porosity as well as fiber geometry. At high flow rates, significant amounts of bead defects can be observed (Supaphol et al. 2012). The applied voltage has been found to influence the macroscale morphology of the nanofibers. The fiber diameter usually decreases using high voltage and low flow rate. The power supply should be adequate to overcome the viscosity and surface tension of the polymer solution to form and sustain the jet from the pipette (Frenot and Chronakis 2003; Ramakrishna et al. 2005; Sharma et al. 2015).

Ambient parameters in the production are likewise important in controlling the formation of nanofibers. Temperature, relative humidity, and air velocity in the electrospinning chamber play a major role in the properties of the polymeric solution and, consequently, modulate the nanofiber formation. It has been reported that high environmental temperature leads to an increase in solvent evaporation rate and as a result thicker nanofibers occur (Esentürk et al. 2016; Melanko et al. 2009; Pelipenko et al. 2015).

Taking all parameters into account, the choice of optimum electrospinning conditions depends on the selected polymer or polymers blend along with the intended use of the resulting nanofibers. By appropriately varying all or some of these parameters, fibers with desired morphologies can be obtained. However, the optimization of the process and system parameters is complex, and keeping close conditions in industrial scale is still a challenge.

5.4 Characterization of Nanofibers

During the preparation of polymeric nanofibers, it is crucial to monitor the basic properties such as nanofiber morphology, molecular structure, and mechanical characteristics. The average fiber diameter, porosity, and hydrophobic property are related to the morphology of the nanofibrous membrane. The molecular structure affects the optical, thermal, and mechanical behavior of electrospun nanofibers. Mechanical properties of nanofibers are important especially in biomedical applications such as wound dressings. The most commonly used techniques for the characterization of nanofibers are summarized in Table 5.2.

The morphological characterization of nanofibrous materials requires a complex approach and evaluation of the results of various methods. SEM, FE-SEM, TEM, and AFM have been used as complementary measurements in morphological characterization of the nanofibrous samples (Viana et al. 2015). SEM is the most used technique to evaluate the fiber diameter, diameter distribution, and surface morphology of prepared nanofibers (Fig. 5.4). FE-SEM is recommended to investigate biodegradable polymer-based nanofibers with poor heat resistance (Melanko et al. 2009; Širc et al. 2012; Vashisth et al. 2016).

The pore size of nanofibers and its distribution can be measured by using electron microscopy techniques and AFM. However porosity cannot be measured by these methods. The most commonly methodology to determine the porosity and pore size of nanofiber scaffolds is using mercury porosimetry (Morie et al. 2016). This method is based on the property of mercury that does not wet the surface of solid materials. During the measurement, mercury is transferred into the sample under vacuum and pressure is applied. Then, the sample porosity is calculated from the mass of mercury penetrated into the pores at highest pressure (Širc et al. 2012). The total pore volume and the total pore area can also be determined by using the same method. The total volume of pores gives foresight about the internal structure of nanofiber mats (Dubský et al. 2012). The major drawbacks of mercury

Table 5.2 Methods for nanofiber characterization

Nanofiber property	Characterization method
Fiber diameter, orientation, structure, morphology	Scanning electron microscopy (SEM), field emission SEM (FE-SEM)
Surface roughness	Atomic force microscopy (AFM)
Internal structure	Transmission electron microscopy (TEM)
Chemical functional groups Polymer–drug secondary interactions	Fourier transform infrared (FTIR) spectroscopy
Crystallographic structure and phase analysis Polymer–drug secondary interactions	X-ray diffraction (XRD) analysis
Porosity and pore size distribution	Mercury porosimetry
Specific surface area	Brunauer–Emmett–Teller (BET) analysis
Mechanical characteristics	Dynamic mechanical analysis (DMA) Tensile strength measurement
Wettability	Water contact angle measurement
Swelling behavior	Swelling index measurement
Thermal behavior	Differential scanning calorimetry (DSC) Differential thermal analysis (DTA) Thermogravimetric analysis (TGA)
Other	Drug entrapment efficiency Drug release study In vitro cell viability study In vitro cell attachment study

porosimetry are its cost and toxicity and the risk of the sample being destroyed at very high pressures.

In order to apply the nanofiber scaffolds for various biomedical applications, an appropriate mat with desirable wettability should be prepared. The hydrophilicity of electrospun nanofibrous membranes can be investigated by water contact angle measurement (Fazli and Shariatinia 2017). Hydrophilic nanofibers show low contact angle, depending on the spreadability of water across surface, while hydrophobic nanofibers show high contact angle because of the minimal contact between water droplet and nanofiber surface (Ramakrishna et al. 2005). Cross-linked nanofiber formulations based on hydrophilic polymers show an increase in the contact angle value as compared to noncross-linked counterparts which reflects the improved aqueous stability of cross-linked nanofibers (Vashisth et al. 2016).

In electrospun nanofiber membranes, the mechanical property is determined by the arrangement and packing characteristic of individual fibers that made up the membrane. As the electrospinning process is influenced by various interrelated and independent variables, it is advisable to perform mechanical testing of the nanofiber membrane when electrospinning process parameters have been altered. Moreover, in order to meet with long-life durability in biomedical applications, the mechanical properties of nanofibrous membranes should be determined. For mechanical characterization, dynamic mechanical analysis (DMA) and tensile strength measurement are the most applied techniques (Ramakrishna et al. 2005).

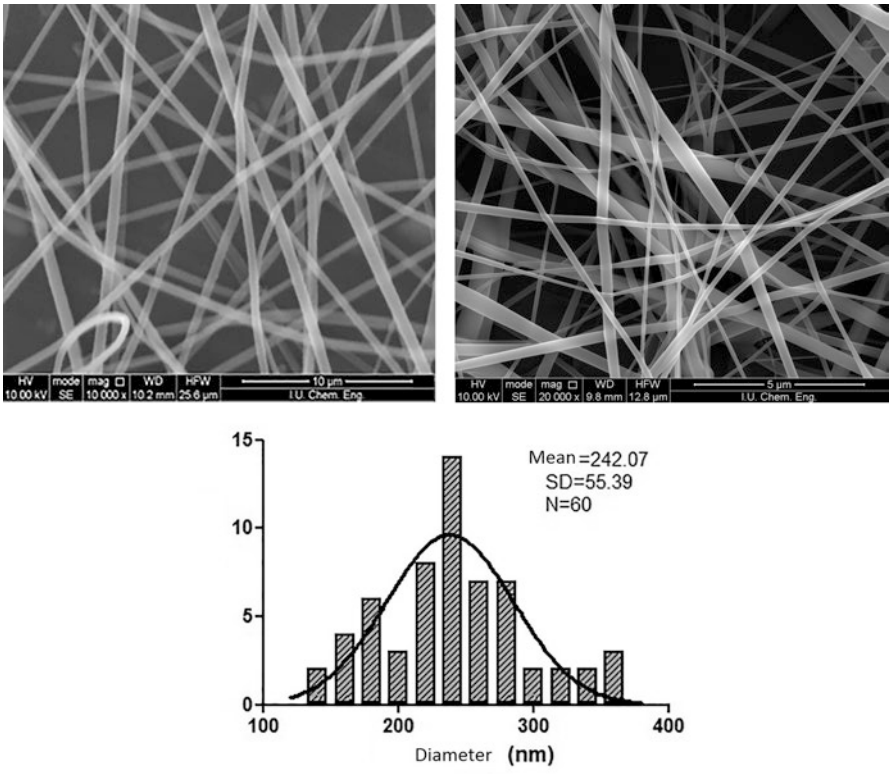

Fig. 5.4 SEM images and mean diameter of PVA/sodium alginate nanofibers (Esentürk et al. 2020)

The structures of the polymer molecules within the nanofibers, polymer–drug compatibility in drug-loaded nanofibers, and nanofiber surface chemistry can be investigated by FTIR spectroscopy. TGA and DSC can be used to study the thermal behavior of the nanofiber composites, whereas the crystallographic structure can be evaluated by XRD analysis (Melanko et al. 2009). The high evaporation rate of the solvents in electrospinning process provides the formation of a solid drug solution in polymer fibers. Therefore, amorphous form of the drug is achieved in the final nanofiber mats (Akduman et al. 2016). Since an amorphous form is thermodynamically less stable than any crystalline form, it can be a challenge to ensure physicochemical stability for the entire shelf life of the drug product (Censi and Di Martino 2015). Especially for hydrophilic drugs which are incorporated into hydrophobic polymer fibers, crystal growth of the drug on the surface of electrospun nanofibers represents a major stability challenge (Kamble et al. 2017; Seif et al. 2015).

Other than these basic physicochemical characterizations, there are a few methods which are also important to characterize nanofiber composites for drug delivery applications. Drug entrapment efficiency and the cumulative in vitro drug release should be determined in drug-loaded nanofibers. The amount of entrapped drug (%,

w/w) in nanofiber mats is usually measured by dissolving the dry mat in the electrospinning solvent. Then the amount of the drug can be measured by using an appropriate method, such as UV spectrophotometer or high-pressure liquid chromatography (HPLC). The mechanism of drug release can be elucidated by transforming and interpreting the in vitro release data into mathematical models. The nanofibers produced by conventional electrospinning from drug–polymer mixed solution usually display burst release behavior because of the short diffusion pathway (Kyziol et al. 2017). The burst release behavior is also associated with the presence of drug on the surface of the nanofibers (Hall Barrientos et al. 2017). This problem can be eliminated by a cross-linking process or by increasing the thickness of the membrane. Zhang et al. (2018) developed hybrid nanofibers for effective drug encapsulation and controlled release. Drug-loaded inorganic nanoparticles have been mixed with PLGA for subsequent electrospinning to form hybrid nanofibers. The preloading of drug molecules within nanocarriers significantly extended the drug diffusion distance; hence the formed hybrid nanofibers displayed reduced burst release profiles. It has been also shown that the electrospinning setup significantly affect the burst release profile of the drugs (Goyal et al. 2016).

The biodegradation profile, cellular compatibility, and cytotoxicity of drug-loaded nanofibers can also be assessed by using various biochemical assays. In industrial-scale production of nanofibers, it is desirable that the main techniques used for the characterization are automated and can be applied online (Pelipenko et al. 2015).

5.5 Polymers Used in Nanofiber Formation

The selection of polymer is of critical importance for the production of nanofibers. The type of the polymer can be chosen depending on the treatment, on the nature of the drug, and on the compatibility with the biological environment (Fig. 5.5) (Pelipenko et al. 2015; Sharma et al. 2015). Although synthetic polymers were the first to be electrospun, biopolymers have gained increasing attention because of their compatibility with biological tissues. Naturally occurring polymers exhibit good biocompatibility and low immunogenicity; hence they have been used widely in wound healing and skin regeneration applications (Erdal et al. 2016; Bhardwaj and Kundu 2010; Zhao et al. 2016). Synthetic polymers can be divided into biodegradable and nondegradable types. They provide many advantages over natural polymers such as wider range of properties, predictable lot-to-lot uniformity, and reliable source of raw materials. The biodegradable synthetic polyesters PLA, PGA, PLGA, and PCL have been commonly used for sustained drug release from nanofibers (Supaphol et al. 2012).

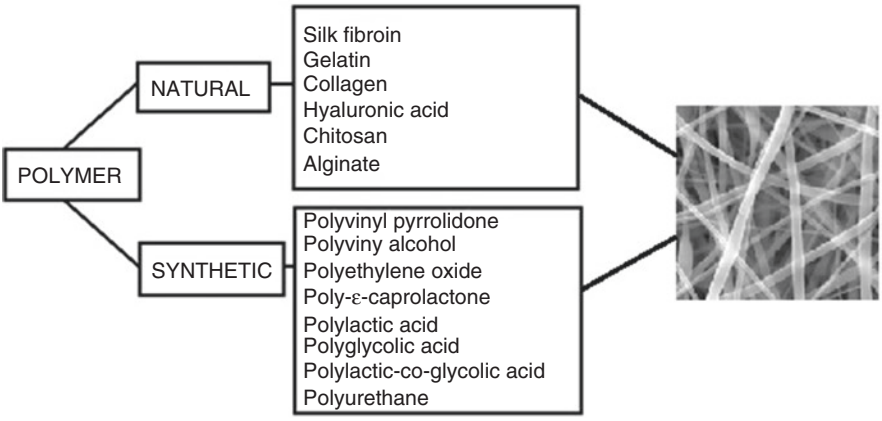

Fig. 5.5 The most widely used natural and synthetic polymers in drug-loaded electrospun nanofibers

5.5.1 Natural Polymers

Natural polymers offer the advantage of being very similar to the macromolecules present in the ECM (Melanko et al. 2009; Sharma et al. 2015). They are usually biodegradable and their biocompatibility is excellent. However, natural polymers often lack the desired physical properties, and they are difficult to electrospin on their own, so they are used mostly in combination with synthetic polymers (Supaphol et al. 2012). The most important biopolymers for the production of drug-loaded electrospun nanofibers are briefly discussed below.

Silk Fibroin Silk is a well-described natural fiber, which has been used in textile industry for ages. Silk fibroin is a main component of silk. It has excellent mechanical strength, and it is biocompatible and biodegradable (Zhao et al. 2016). Electrospun fibers from silk fibroin were found to promote cell adhesion and proliferation, and they found place in tissue engineering, wound healing, and drug delivery studies (Bhardwaj and Kundu 2010; Ramakrishna et al. 2005). Silk protein is water soluble, and therefore cross-linking treatments have been used to increase its water resistance (Kluge and Mauck 2012).

Gelatin Gelatin is obtained by partial hydrolysis of collagen. It is widely used in the pharmaceutical industry and biomedical fields due to its nontoxic nature and biodegradability (Erdal et al. 2016). Gelatin scaffolds have been engineered to facilitate the regeneration of bones, skin, muscles, and nerves (Gomes et al. 2015). However, the mechanical strength of gelatin nanofibers is low, and therefore cross-linking treatments have been used to increase their water resistance (Laha et al. 2016; Zhao et al. 2016). The blends of gelatin with natural or synthetic polymers have been used to improve the mechanical properties of nanofibers (Aldana and Abraham 2017; Laha et al. 2016). Gelatin/poly-ε-caprolactone (PCL) composite

systems showed better mechanical strength and wettability compared with gelatin or PCL alone (Vasita and Katti 2006). Gelatin nanofibers produced by needleless electrospinning accelerated wound healing in full-thickness wound model in rats (Dubský et al. 2012).

Collagen Collagen is the most abundant protein in animals and in the human body. It is a major component of the skin, cartilage, bone, tendon, and teeth. In many native tissues, type I and III collagen are the principal structural elements of the ECM (Bhardwaj and Kundu 2010; Zhao et al. 2016). Collagen as a biopolymer has been used in wound dressings. It has also been tested in skin, bone, tendon, and ligament tissue engineering. In vitro studies had shown that cells respond positively to tissue scaffold made of electrospun collagen fibers (Ramakrishna et al. 2005). The nanofibers based on collagen have been shown to be compatible with a number of cell types and offer a suitable environment for cell growth (Vasita and Katti 2006). But, electrospinning of pure collagen is difficult, and usually a stabilization procedure by cross-linking with formaldehyde or glutaraldehyde is needed (Erdal et al. 2016; Pelipenko et al. 2015).

Hyaluronic Acid Hyaluronic acid is a linear polyanionic glycosaminoglycan, and it is a natural component of the ECM of connective tissues (Bhardwaj and Kundu 2010). Due to its unique rheological properties and biocompatibility, hyaluronic acid has been used extensively in many biomedical applications such as ophthalmology, medical implants, and drug delivery (Ramakrishna et al. 2005). Hyaluronic acid nanofibers have been tested in the development of modern wound dressings. But electrospinning of hyaluronic acid alone does not allow a consistent production of fibers, and the main drawbacks of hyaluronic acid nanofibers have been reported as poor cell adhesion, mechanical instability, and rapid degradation in vivo (Pelipenko et al. 2015; Vasita and Katti 2006).

Chitosan Chitosan is a biodegradable and biocompatible polymer. Chitosan has been chosen for infection-related wound healing studies in a majority because of the rich hydrogen bonds between chitosan chains that allow a favorable swelling ability to the polymer (Wang et al. 2017). Chitosan-based nanofibers were applied for tendon, bone, and cartilage tissue engineering (Bhardwaj and Kundu 2010; Erdal et al. 2016; Pelipenko et al. 2015). Although it is possible to electrospun pure chitosan, the nonaqueous solvents, which should be used in the electrospinning process, possess very harmful characteristics. Therefore, blends of chitosan with synthetic polymers such as polyvinyl alcohol (PVA) or polyethylene oxide (PEO) have been used in order to improve the spinnability of chitosan (Zhao et al. 2016).

Alginate Alginate is a water-soluble, natural linear polysaccharide obtained from brown seaweed. It is a biopolymer which plays an important role in the design of controlled drug delivery formulations, and it shows great potential as a scaffold material in tissue engineering (Erdal et al. 2016). Alginate-based nanofiber wound dressings have been widely studied due to the specific characteristics of alginate,

including biocompatibility, nonimmunogenicity, and large water-absorbing capacity (Pelipenko et al. 2015; Zhao et al. 2016). Alginate alone could not electrospun because of its poor mechanical strength and processing difficulties. Therefore it is usually blended with appropriate polymers with a high molecular weight (Fu et al. 2016; Kyziol et al. 2017).

5.5.2 Synthetic Polymers

A wide variety of synthetic polymers has been used to fabricate nanofiber sheets by electrospinning technique (Vasita and Katti 2006). Especially, the water-soluble synthetic polymers polyvinylpyrrolidone (PVP), PVA, and PEO have been accepted as ideal candidates to fabricate nanofibers for use as drug carriers (He et al. 2014). The composite nanofibers composed of synthetic and natural polymer blends combine the favorable biological characteristics of natural polymers and the mechanical performance of the synthetic ones (Aldana and Abraham 2017).

Polyvinyl Alcohol (PVA) PVA is a nontoxic, highly hydrophilic, biodegradable, semicrystalline polymer with adhesive properties (Fu et al. 2016). It has good chemical and physical stability and has the ability to form fibers, films, and membranes (Wali et al. 2018). PVA has been widely used to produce nanofiber mats due to its good spinnability (Arthanari et al. 2016; Viana et al. 2015). The morphology of electrospun PVA nanofibers has shown to be affected by concentration of the polymer solution, ionic salt addition, applied electric potential, and pH (Ngawhirunpat et al. 2009). The elastic properties of PVA nanofibers have been found suitable for regeneration of soft tissues such as skin (Pelipenko et al. 2015). The fabrication of polyblend PVA–natural polymer nanofibers provided improved mechanical strength, bioactivity, and degradation profile to the end product and introduced scaffolds with desired properties for specific tissue regeneration and drug delivery applications (Zanin et al. 2011; Zamani et al. 2013).

Polyethylene Oxide (PEO) PEO is a unique class of nonionic water-soluble, biodegradable polymer with a linear structure and good spinnability. It shows excellent biocompatibility and very low toxicity (Kyziol et al. 2017). PEO nanofibers can be stabilized physically. Blending of PEO with biopolymers has become a popular tool for overcoming the processing limitations of less soluble or less available materials (Kluge and Mauck 2012). In the electrospinning field, PEO and PVA are often used in the preparation of nanofibers from their blend solutions (Fu et al. 2016). They are also often added to chitosan, alginate, and hyaluronic acid which are difficult to be electrospun alone (Pelipenko et al. 2015).

Poly-ε-caprolactone (PCL) PCL is an FDA-approved semicrystalline and biodegradable hydrophobic polyester which is commonly used in electrospun nanofibers for controlled release (Hall Barrientos et al. 2017). It is characterized by a high

plasticity and a slow degradation rate resulting from the hydrolysis of its ester linkages (Anjum et al. 2017; Gomes et al. 2015). The mechanical properties of PCL as well as its spinnability are excellent. PCL nanofibers are stable in an aqueous environment, but organic solvents are needed for their production (Pelipenko et al. 2015; Chou et al. 2015). Due to the limited cell specificity and high hydrophobicity of PCL, incorporation of pore-generating polymers, like PEG or gelatin, into PCL nanofibers has been shown to increase the biofunctionality (Ravikumar et al. 2017; Wang et al. 2016). Surface treatment of PCL nanofibrous scaffolds with ethanol can create a hydrophilic surface which serves for efficient cell attachment (Kluge and Mauck 2012).

Polyvinylpyrrolidone (PVP) PVP is a hydrophilic, nontoxic, biocompatible synthetic polymer (Maslakçı et al. 2017). Electrospun PVP nanofibers have been tested as a drug delivery systems and wound dressings. The water absorbability and dissolution properties of PVP nanofibers have been found to improve the dissolution profile of incorporated drugs (Yu et al. 2010).

Polylactic Acid (PLA), Polyglycolic Acid (PGA), and Polylactic-co-glycolic Acid (PLGA) The most commonly used synthetic polymers for three-dimensional tissue scaffolds are saturated biodegradable polyesters, including PLA and PGA, as well as PLGA copolymers. PGA is a hydrophilic and highly crystalline polymer with a relatively fast degradation rate. Although structurally very similar to PGA, PLA exhibits different chemical, physical, and mechanical properties. The nanofibers obtained from PLA, PGA, and PLGA have been found mechanically stable, but organic solvents are needed for their production (Kluge and Mauck 2012; Said et al. 2011).

Polyurethane (PU) PU is one of the most widely used polymers in biomedical applications especially those in contact with blood (Ramakrishna et al. 2005). PU nanofibers possess excellent mechanical properties, and they have been widely used in wound healing applications due to their oxygen permeability and barrier properties (Akduman and Kumbasar 2017).

5.6 Electrospun Nanofibers in Dermal Drug Delivery

Among the various applications of electrospun nanofibers, drug delivery plays a key role for biomedical applications. The advantages of using nanofibers in drug delivery can be considered as (a) high drug loading and encapsulation efficiency, (b) ability to modulate drug release, (c) the possibility to use a wide variety of both natural polymers and synthetic polymers, and (d) the high surface area-to-volume ratio that facilitates mass transfer and efficient delivery of both hydrophilic and hydrophobic drugs (Goyal et al. 2016; Chou et al. 2015; Manuel et al. 2016; Melanko et al. 2009). Small molecular weight drugs, proteins, DNA, genes, and

vaccines can be incorporated to the electrospun nanofibers for specific therapeutic purposes (Sharma et al. 2015; Zamani et al. 2013). During the last several decades, polymeric nanofibers have been explored as controlled drug delivery systems for dermal, transdermal, oral, oromucosal, parenteral, and ocular routes (Pelipenko et al. 2015). Recently, electrospun nanofibers have gained more popularity for the topical and transdermal drug delivery and wound dressing applications.

The use of the skin as a drug delivery route for both topical and systemic therapy is a noninvasive approach for administration of drugs. However, the remarkable barrier properties of the skin, especially of its outermost layer, stratum corneum, pose a significant challenge to administering medications via the skin either for local cutaneous effects or as systemic therapy. In order to deliver drugs through the skin, most compounds require various degrees of permeation enhancement. Nanofiber-based dermal delivery systems have been considered as tool to improve the solubility of active agents and thereby enhancing drug release and skin permeation characteristics (Kamble et al. 2017). Table 5.3 represents a selection of scientific studies dealing with the nanofiber-based dermal drug delivery in the last decade. As evident from the table, drug-loaded nanofibers have especially attracted great attention for their use in wound healing applications.

PCL poly(Ɛ-caprolactone), *PEO* poly(ethylene oxide), *PVA* poly(vinyl alcohol), *PVAc* poly(vinyl acetate), *PNIPAM* poly(-N-isopropylacrylamide), *PGS* poly(glycerol sebacate), *CA* cellulose acetate, *PVP* poly(vinylpyrrolidone), *SA* sodium alginate, *PLA* poly(lactic acid), *PLGA* poly(lactic-co-glycolic acid), *PU* poly(urethane), *HPC* hydroxypropyl cellulose, *PMMA* poly(methyl methacrylate)

Wound healing is essential for the restoration of the skin barrier after injury. It comprises of healing of dermal and epidermal tissues by their regeneration. During the wound healing process, the cells at the wound edge proliferate and migrate, leading to reepithelization of the wound surface. This is a complex and dynamic process involving five stages: hemostasis, inflammation, proliferation, migration, and maturation (Stamm et al. 2016). During this period the disturbance of wound healing can be caused by several factors, such as infection, excess of inflammatory cytokines, and necrosis (Anjum et al. 2017; Garcia-Orue et al. 2017).

The prevalence of chronic dermal wounds has increased in recent years due to the increase of the high-risk population including elderly, diabetics, and obese. The inflammation stage in chronic wounds proceeds too long, resulting in wounds that remain open for months to years. Therefore, the treatment of chronic wounds is still a significant clinical challenge (Goyal et al. 2016). Current studies to overcome this problem are focused on the development of new therapeutic approaches. Recently, the electrospun nanofibrous wound dressings have been shown to be able to enhance the wound healing process because of their potential to mimic the structure and biological function of dermal ECM (Anjum et al. 2017; Mickova et al. 2012; Wang et al. 2017).

The unique architectural properties like nanoscale morphology, porous structure, and flexibility of electrospun nanofibers make them a suitable option for developing novel wound dressings (Garcia-Orue et al. 2017). The very high porosity of the nanofibrous membranes allows cell respiration and gas permeation and prevents the

Table 5.3 Nanofiber-based dermal drug delivery studies conducted in the last decade

Polymer or polymer blend	Drug	Aim	Author and Year
PU	Doxorubicin	Wound healing	Kiliç et al. (2018)
Chitosan/PVA	Tetracycline hydrochloride	Wound healing	Alavarse et al. (2017)
Chitosan/PVA	Ampicillin	Wound healing	Wang et al. (2017)
Chitosan/PEO	Cefazolin and cefazolin loaded nanoparticles	Wound healing	Fazli and Shariatinia (2017)
Chitosan/PCL	Ferulic acid and resveratrol	Wound healing	Poornima and Korrapati (2017)
Nanochitosan/PCL	Curcumin	Wound healing	Cr et al. (2018)
Gelatin	*Centella asiatica* extract	Wound healing	Yao et al. (2017)
Gelatin/silk fibroin	Thyme essential oil and doxycycline	Wound dressing	Dadras Chomachayi et al. (2017)
Gelatin/PCL	Cerium oxide nanoparticles	Wound healing	Rather et al. (2017)
Gelatin/PGS	Ciprofloxacin	Wound healing	Shirazaki et al. (2017)
Gelatin/PVA/PCL	Bromelain and salvianolic acid	Wound healing	Shoba et al. (2017)
SA, chitosan, PCL, collagen, PEO	Doxycycline	Wound healing	Tort et al. (2017)
SA/PEO	Ciprofloxacin	Wound healing	Kyziol et al. (2017)
PLGA	Recombinant human epidermal growth factor and *Aloe vera*	Wound healing	Garcia-Orue et al. (2017)
PLGA	Growth factors	Skin tissue regeneration	Lee et al. (2017)
PLA	Ibuprofen	Wound healing	Mohiti-Asli et al. (2017)
PVP/dextran	Ibuprofen and acetylsalicylic acid	Antimicrobial wound dressing	Maslakçı et al. (2017)
PVA/SA	Dexpanthenol	Wound healing	Tamizi et al. (2017)
PVA/calcium alginate	Papain	Wound healing	Dutra et al. (2017)
Eudragit RL/RS	Gentamicin sulfate and recombinant human epidermal growth factor	Wound healing	Dwivedi et al. (2018)
PCL–PEG–PCL triblock copolymer	Magnetic iron oxide nanoparticles	Skin tissue engineering	Zhang et al. (2017)
PCL/PVA	Silver sulfadiazine	Antimicrobial wound dressing	Mohseni et al. (2016)
PCL/gum tragacanth	Curcumin	Wound healing	Ranjbar-Mohammadi et al. (2016)
PCL/hyaluronan	Epidermal growth factor	Wound healing	Wang et al. (2016)
PVP	Chloramphenicol and suberin fatty acids	Wound healing	Tamm et al. (2016)

(continued)

Table 5.3 (continued)

Polymer or polymer blend	Drug	Aim	Author and Year
PMMA/PVA	Ciprofloxacin	Wound healing	Zupančič et al. (2016a)
Chitosan/PEO	Metronidazole	Wound healing	Zupančič et al. (2016b)
PU/dextran	β-Estradiol	Post-menopausal wound care	Unnithan et al. (2015)
Collagen/hyaluronic acid	Multiple angiogenic growth factors	Wound healing	Lai et al. (2014)
Chitosan/PEO	Blood-derived growth factors	Wound healing	Bertoncelj et al. (2014)
SA/PVA	Ciprofloxacin	Wound healing	Kataria et al. (2014)
SA/PVA	Gatifloxacin	Wound healing	Arthanari et al. (2016)
PVA/PCL	Horseradish peroxidase	Wound healing	Mickova et al. (2012)
PVA/PVAc	Ciprofloxacin	Wound healing	Jannesari et al. (2011)
PLGA	Fusidic acid	Wound dressing	Said et al. (2011)
PLGA	Rhodamine B	Tissue engineering scaffold	Liao et al. (2008)
PU/HPC	Donepezil hydrochloride	Transdermal drug delivery system	Gencturk et al. (2017)
Chitosan/ phospholipid	Curcumin, diclofenac, and vitamin B12	Transdermal drug delivery system	Mendes et al. (2016)
PLGA	Daidzein-loaded lipid nanocarriers	Transdermal drug delivery system	Song et al. (2016)
PVP	Curcumin	Transdermal drug delivery system	Wang et al. (2015)
PVA	Prazosin hydrochloride	Transdermal drug delivery system	Shen et al. (2014)
CA/PVP	Ibuprofen	Transdermal drug delivery system	Shi et al. (2013)
PVA/CA	Capsicum extract	Transdermal drug delivery system	Opanasopit et al. (2013)
PVA	Meloxicam	Transdermal drug delivery system	Ngawhirunpat et al. (2009)
PU	Naproxen	Topical drug delivery system	Akduman et al. (2016)
PCL	Linezolid	Topical antibacterial	Tammaro et al. (2015)
PVA/PNIPAM	Levothyroxine (T_4)	Sustained topical delivery of T_4	Azarbayjani et al. (2010)

wound from bacterial penetration and dehydration (Mickova et al. 2012). Most of the studies have shown that electrospun nanofibrous scaffolds might be capable of supporting cell adhesion, proliferation, and maturation (Anjum et al. 2017; Frenot and Chronakis 2003). Generally, the hydrophilic natural polymers provide sites for cell adhesion, while synthetic polymers add mechanical strength and slow the degradation rate (Gomes et al. 2015). Upon proper polymer selection nanofibers can also provide an optimum barrier for appropriate healing of the wound (Kamble et al. 2017; Sharma et al. 2015; Zanin et al. 2011). The local delivery of drugs via bioactive nanofiber mats is one of the effective approaches to accelerate the wound healing process more efficiently (Kataria et al. 2014). Drug-loaded nanofibers composed of chitosan–PEO (Bertoncelj et al. 2014; Fazli and Shariatinia 2017), chitosan–PVA (Alavarse et al. 2017), chitosan–PCL (Poornima and Korrapati 2017), and gelatin–PCL (Rather et al. 2017) blends or hydrophilic synthetic polymers such as PVA (Shoba et al. 2017), PLA (Mohiti-Asli et al. 2017), and PVP (Tamm et al. 2016) have been specifically engineered to promote wound healing and skin regeneration.

5.7 Conclusions

Among the different nanotechnology-based drug delivery systems, electrospun nanofibers have been considered as promising novel carriers due to their efficiency on drug delivery in a controlled manner. The nanofiber scaffolds composed of biocompatible polymers are widely studied as effective alternatives for skin wound treatment and dermal drug delivery. On the other hand, the scale-up to industrial production and the clinical studies of drug-loaded nanofibers are still limited. Further studies have to be performed to overcome the technical challenges to ensure industrial production of uniform nanofibers. Furthermore, the effect of drug release kinetics on therapeutic efficiency as well as possible toxic effects should be clarified by in vivo studies.

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Chapter 6
Enzyme-Responsive and Enzyme Immobilized Nanoplatforms for Therapeutic Delivery: An Overview of Research Innovations and Biomedical Applications

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Abstract Enzymes facilitate physiological function but are dysregulated in disease-associated environments. The utilization of the altered enzyme activity for therapeutics exhibits enormous promise. The amalgamation of nanomaterials with such enzymatic responses as enzyme responsive and immobilized nanoplatforms has found growing applications in therapeutic delivery of enzyme-triggered nanomedicine. However, the key problem is the low stability and high immunogenicity of these nanobiocatalysts that restricts their use in therapy. These limitations can be overcome through designing, functionalization, and engineering of smart biocompatible nanocarriers that improves stability and reduces immunogenic responses leading to more efficient and targeted delivery of drug.

In enzyme-responsive drug delivery, the affected tissues secrete disease-specific enzymes that help in the release of drugs from the coated nanocarriers at the diseased tissue. Apart from this, enzymes can be immobilized that act as drugs for regulation of diseases. In this chapter, initially the types of nanomaterials for immobilization of enzymes with improved biomedical application potential highlighting their effectiveness as new tools for future therapeutics are discussed. Subsequently, protease, lipolytic and oxidoreductase-responsive nanocarriers, and the salient features of enzyme immobilized nanoplatforms for biomedical applications are explored. Moreover, applications of the immobilized enzymes for treatment of various diseases like cancer, wound healing, alcohol intoxication, and Gaucher's disease along with the challenges and suggestions for future research are delineated. Overall, this chapter reviews the effectiveness of enzyme-responsive and immobilized nanomaterials as emerging tools for future therapeutics.

Keywords Nanoparticle · Enzyme responsive · Immobilization · Therapeutic delivery · Biomedical applications

6.1 Introduction

Life depends upon numerous synergistic metabolic processes that rely on protein machinery of the organism. Enzymes are the workhorse molecules of life that keeps the organisms running by getting integrated into various metabolic pathways. Various diseases disrupt these metabolic pathways, and the enzymes involved in them are the biorecognition molecules that play an imperative role in the regulation of diseases (DeBerardinis and Thompson 2012). Enzymes have the potential of combating as antidotes or therapeutic agents. So currently, enzyme therapy is an effective and efficient way to treat diseases (Chowdhury et al. 2017). Nevertheless, low stability and high immunogenicity are the major limitations of enzyme therapy that can be resolved by enzyme-responsive and immobilized nanoenzyme delivery systems. The major advantages of enzyme immobilization for disease therapeutics include increased stability, decreased immunogenic responses, enhanced functional efficiency, minimum therapeutic time, continuous use of enzymes, etc. (Bosio et al. 2015). These benefits of enzyme immobilization can employ a wide range of nanomaterials to make nanoenzyme complex as an avenue for therapeutic delivery. The additional approach of enzyme-activated (or responsive) drug carriers releases the drug molecules to the specific targeted tissues (Hu et al. 2014). Due to the presence of specific enzyme in the carrier reacting with the substrate or the triggered molecule at the site of infection, the drug is eventually released. This is advantageous as the rate of release of drugs can be adjusted to the changing physiological conditions (Shukla et al. 2016). Thus, research have propelled toward enzyme-mediated nanotherapy techniques for targeted treatment of diseased cells without damaging healthier tissues of the organisms referred to as tissue-specific targeted drug delivery.

For successful and effective drug delivery, various nanosystems with varying sizes are applied leading to efficacious therapy. But, the problems encountered in efficacious nanoconjugated drug delivery are optimized synthesis of drug nanocarriers, sustained targeted drug delivery at the site of infection, immunological rejection, and toxicological effects in the host. Even though nanomedicine can be efficient in in vitro or ex vivo studies, it is important to evaluate the different platforms that are decided upon multiple considerable factors such as required size of nanoparticles, surface modification (hydrophobic or charged), permeability to the biological barriers, biodegradable, biocompatible, nontoxicity, controlled drug release at desired site, site-specific drug targeting, and nonresponsive to the host immune system required for in vivo delivery (Li et al. 2012; Shukla et al. 2016). Most importantly, site-specific drug targeting is only achieved by tagging the nanoassemblies with receptor-specific ligands for internalization into specific cells (Xu et al. 2013). Many biocompatible nanosupports comprising biological entities such as protein, polysaccharide, DNA, etc. are used for diseases like cancer, wound healing, inflammation, infectious diseases, myocardial infarction, neurodegeneration disorders, etc. (Kalam et al. 2017; Chowdhury et al. 2017). Thus, nanotherapeutic platforms have provided the opportunities to get direct access to the diseased cells selectively with improved drug localization and cellular uptake.

The central tenet of this chapter is to demonstrate current research innovations and biomedical applications of both enzyme-responsive nanoparticles and immobilized enzymes as drug for biomedical therapeutics. The present chapter discusses various nanomaterials employed for immobilization followed by enzyme-responsive nanocarriers for effective drug release, the key parameters for nanodesign consideration for therapeutic efficacies, and finally focused upon biomedical therapeutics using immobilized enzymes for various diseases. At last, the challenges regarding nanomaterial designing, biological barriers, their toxicological and immunological effects, tissue-specific delivery, their pharmacokinetics parameters, etc. along with the suggestive strategies to resolve these challenges are being discussed.

6.2 Types of Nanomaterials for Immobilization of Enzymes

Immobilization of enzymes on to the selected solid support refers to the interaction between them with specific chemical bonds that changes the physical, biochemical, and kinetic properties of biocatalysts. However, immobilization can sometimes alter the kinetic parameters such as K_m, V_{max}, turnover rate, and catalytic efficiency (Mohamad et al. 2015). Immobilization of enzymes makes the enzyme more robust than that of their native form by increasing enzyme stability in terms of extreme pHs and elevated temperatures, in solvents, and at higher ionic strengths (Mohamad et al. 2015). From the therapeutic point of view, immobilized enzymes have advantageous attributes that include resistance to autolysis from hydrolases, reduce shearing effects, reusability of the biocatalyst in the living system, prolonged catalytic half-life, and extended half-life of enzyme in the in vivo system.

There are several principal techniques for immobilization of enzymes like entrapment, adsorption, covalent, and cross-linking for in situ and ex situ conjugation and purification (Mohamad et al. 2015). The enzyme entrapment method involves trapping of enzyme within the matrix in such a way that the enzyme remains entrapped but allows the substrate and products to pass through (Aehle 2007). In physical adsorption method, the enzymes are being reversibly adsorbed or attached onto the support material through van der Waals interactions, hydrophobic interactions, hydrogen bonds, etc. (Jegannathan et al. 2008). Crosslinking includes formation of intermolecular crosslinkages between enzyme molecules and matrix by means of reagents such as glutaraldehyde (Sheldon 2011; Saha et al. 2018). Lastly, the covalent binding immobilizes enzymes irreversibly via the side chains of surface-exposed residues like ϵ-amino group of lysine, thiol group of cysteine, and carboxylic group of aspartic and glutamic acids (Singh and Lillard 2009). In general, enzyme immobilization solid supports can be grouped into polymeric-based nanoparticles, metal-based nanoparticles, silica-based nanoparticle, carbon-based nanoparticles, liposomes, crosslinked enzyme aggregates, and hybrid nanoparticles.

6.2.1 Polymeric Nanoparticles

The use of polymeric nanoparticles is beneficial for optimal drug delivery in varied drug dosages as they are more biocompatible, increase drug efficacy to the target tissue, reduce side effects, and improve patient compliance. They are considered as promising approach to augment chemical drug delivery systems (Łukasiewicz et al. 2015). Enzyme-responsive polymeric nanoparticles take advantages of overexpressing enzymes in the disease tissues such as Matrix metalloproteinases (MMPs) in cancer and alter polymeric material chemical structures in response to these specific enzymes. Various polymeric nanoparticles are exploited with drugs and therapeutic enzymes for diseases such as cancer, diabetes, etc. (Hu et al. 2012). Several natural polymers (alginate, carrageenan, chitosan, cellulose, chitin, collagen, starch, sepharose, etc.) can be used as support materials but are difficult to tailor into nanosize (under 200 nm) and are generally crosslinked with encapsulated drugs (Meryam Sardar 2015). So, apart from natural polymers, different synthetic polymeric materials [poly(cyanoacrylate), polyanhydride, poly(e-caprolactone), poly(lactic acid) (PLA), poly(lactide-co-glycolic acid) (PLGA)] are also used for immobilization (Choi and Ryoo 2003). Gu et al. (2009) developed enzymatically degradable peptide polymeric nanocapsules that contained functional apoptosis inducing target protein Caspase-3 (CP3). Upon enzymatic degradation, the nanoparticle dissociated and released protein CP3 leading to apoptotic hallmarks of HeLa cells as shrinking of cells after 24 h (Gu et al. 2009). The problem encountered is aggregation of these polymeric nanocarriers that can be overcome by surface modification leading to improved surface to surface interfacial interactions. For surface modification of various nanoparticles, polyethylene glycol (PEG) is used very vigorously nowadays because of its unique properties. PEG is an (i) inert hydrophilic polymer, (ii) has steric hindrance, (iii) prevents protein binding, (iv) reduces immune responses, and (v) enhances half-life of drugs in the in vivo system (Hu et al. 2012). Wong et al. (2011) employed PEG-coated enzyme-degradable nanoparticles of type A gelatin which shrunk from 100 to 10 nm after exposed to overexpressed MMP-2 at the site of tumor microenvironment. In addition to polymeric nanoparticle shrinking, enzyme responses can also increase the size of nanoparticle by cleaving the substrates (Wong et al. 2011). Besides, micelles can also be prepared by integration of hydrophobic block and a hydrophilic peptide into copolymers which can be employed for easy diffusion into the tumors for gaining access to dysregulated enzymes (Akagi et al. 2007). Additionally, natural or synthetic hydrogels or cryogels can be used for enzyme immobilization, for example, polyvinyl alcohol (PVA) cryogels are widely used for whole cells and enzyme immobilization (Homaei et al. 2013). Moreover, a wide range of biopolymers, preferably, water-insoluble polysaccharides like starch, agarose, cellulose, carragenans, chitosan, etc., has been used as supports for enzyme immobilization (Mohamad et al. 2015). Further development of new polymeric-based nanoparticle synthesis will pave a path for evaluation of smart drug delivery.

6.2.2 Metallic Nanoparticles

Metal-based nanoparticles are used for various biomedical applications as they affect immune responses in many organisms. They are significantly used for their different physicochemical properties (smaller size, elemental composition, charge, shape, surface area, solubility, crystallinity, and easy cellular uptake for intracellular distribution) than their conventional bulk materials but are found to be toxic to the physiological environment as they trigger both the innate and adaptive immune responses (Luo et al. 2015). Metal-based nanoparticles like that of silver nanoparticles (AgNPs), gold nanoparticles (AuNPs), and other metal oxide nanoparticles (iron oxide, zinc oxide, titanium dioxide, and quantum dots) are employed for various biomedical and industrial applications (Homaei et al. 2013; Luo et al. 2015). Zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles are used in sunscreens and cosmetic products, and AgNPs are used in antibacterial agents, paints and printer inks, textiles, and detergent applications (Lu et al. 2015). Datta et al. (2017) reported effective anticancer and antimicrobial activity of enzyme-responsive adenosine triphosphate functionalized silver nanoparticles with excellent stability in normal physiological environment (Datta et al. 2017). Development of metallic nanoparticles with improved biomedical application potential highlights their effectiveness as new tools for future therapeutics.

6.2.3 Silica-Based Nanoparticles

Various inorganic solid supports of nanosizes such as alumina, mesoporous silicas, and zeolites have been used for immobilization of enzymes (He and Shi 2011; Peng et al. 2014). Exhaustive research has been performed to use silica-based nanoparticles (SiNPs) in disease diagnostics and drug delivery applications due to their higher mechanical strength, eco- and solvent-friendly, and increase longevity in the in vivo system (He and Shi 2011; Jo and Ban 2016). Moreover, the silica gel can be tuned to required morphology, pore structures, and microchannels (He and Shi 2011). These nanoparticles are considered beneficial as they opened innovative prospects in enzyme supporters for delivery, biosensors development for diagnosis, controlled drug release at the target tissues, and intracellular uptake for biodistribution (He and Shi 2011; Peng et al. 2014; Jo and Ban 2016). Park et al. (2009) constructed an enzyme-responsive porous SiNPs with cyclodextrin gatekeepers, multifunctional stalk structures, and fluorescence probes at nanoparticle surfaces to release drug loads in response to α-amylase and lipase at targeted tissues (Park et al. 2009). Yu et al. (2012) evaluated in vivo toxicity of enzymes-immobilized SiNPs for subcutaneous and oral administration routes which requires high and repeated doses to achieve efficient disease treatment. The effective progress in the field of biodegradable SiNPs may open up new avenues for designing of drug carriers with improved biosafety.

6.2.4 Carbon-Based Nanoparticles

A wide variety of carbon nanomaterials based on carbon allotropes (such as carbon nanotubes, nanohorns, and nanodiamonds) known for versatile physicochemical and structural properties are currently being explored for clinical applications (Liu et al. 2011). They are well-thought-out as novel and innovative tools for in vivo delivery of therapeutic cargo molecules. Graphene is a novel member of carbon nanoparticle family. The immobilization of enzymes onto graphene oxide sheets leads to improved conformation of enzymes for better catalytic performance (Zhang et al. 2010). It is reported that graphene oxide along with near-infrared laser irradiation can be used for photothermal treatment of Alzheimer's disease (Li et al. 2012; Yang et al. 2013). Further, recent evolvement in drug delivery fields based on mesoporous carbon nanomaterials (MCNs) results in immediate, sustained, controlled, and targeted delivery of the drug. MCNs have numerous biomedical applications including photothermal therapy, biotherapeutic agent delivery, mesoporous carbon-assisted bioimaging, etc. (Zhao et al. 2017). For instance, Li et al. (2015) designed enzyme and glutathione dual-responsive drug release MCNs that were functionalized with hyaluronic acid for chemophotothermal therapy. The system was found to be sensitive to both intracellular hyaluronidase and GSH-degrading enzyme to control drug release (Li et al. 2015). Thus, the carbon-based nanomaterials have promising potential for drug or gene delivery.

6.2.5 Liposomes

Liposomes are considered as an excellent smart enzyme delivery nanoplatform for therapeutics due to their biocompatibility and ease of fabrication (Liu et al. 2016). They have advantage of improved delivery efficiency and therapeutic efficacy in terms of their suitable sizes, unique chemical properties, multiple functionality, large surface area to volume ratio, diversity in structure, and receptors recognition ligands to combat various diseases (Akbarzadeh et al. 2013). They are also integrated with the biorecognition elements that are responsive to enzyme overexpressed at the site of diseased tissue (Liu et al. 2016). The release of cargo in enzyme-responsive liposomes occurs through various degradation mechanisms like lipid bilayer perturbation; degradation of coated polymer from the surface and leading to cellular uptake; lipopeptide or lipopolymer cleavage in the lipid bilayer; and prodrug activation in the liposomes (Fouladi et al. 2017). Cummings et al. (2000) reported phospholipase A2 (PLA_2)-mediated hydrolysis of phospholipids at the sn2-fatty acyl ester position that produced free fatty acid and lysophospholipid which was incorporated into liposomes leading to disruption of integrity of the lipid bilayer and released the encapsulated payloads (Cummings et al. 2000). Similarly, Terada et al. (2006) also constructed the PEGylated, galactosylated liposome having protease-specific substrate peptide and conjugated with

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine which showed that liposome did not enter into normal hepatocytes but in upregulated MMP-2 hepatocytes by enzymatic catalyzed interactions and delivered a payload (Terada et al. 2006). Optimal site-specific delivery rather than site-avoidance delivery is the characteristics of liposomal-based drug delivery system, and the developments of fusogenic liposomal-based drug delivery will allow a larger variety of agents to be delivered.

6.2.6 Crosslinked Enzyme Aggregates (CLEAs)

CLEAs are mostly active immobilized enzymes forms and have controllable particle sizes (varying from 500 to 100 μm), operational stability and performance, ease of recycling, and improved productivities used for various biomedical and industrial applications (Sheldon 2011; Islan et al. 2014). They are synthesized by adding salts and organic solvents or nonionic polymers and by physical aggregation. However, they have inherent disadvantage of perturbation by denaturation of tertiary structure and reduction in the enzyme catalytic activity when linked through covalently crosslinker (Sheldon 2011). The penicillin acylase CLEAs are used for large-scale antibiotic production (Cao et al. 2000). In another research, Islan et al. (2014) synthesized novel *Sphingobacterium multivorum*-derived alginate lyase CLEAs with low methoxylated pectin and glutaraldehyde as crosslinker that were used for cystic fibrosis oral therapy (Islan et al. 2014). As a whole, CLEA technology has turned enzymes into a useful tool for the biocatalysis processes and warrants further studies to improve CLEA's performance and bringing up new crosslinking agents.

6.2.7 Hybrid Nanoparticles

A variety of emerging enzyme-responsive hybrid nanoparticles have gained increasing attraction as new delivery platform like lipid–polymer, gel–lipid, silica–polymer, iron oxides–polymer hybrid nanoparticle, etc. (Hoskins et al. 2012; Jiang et al. 2014). They can be engineered to increased surface area-to-volume ratio, controlled morphology, mechanical stability, biocompatibility, biodegradability and biodistribution. Lipid–polymer hybrid nanoparticles have efficient stability and biocompatibility along with in vivo cellular delivery capacity than any other nanoparticles since they exhibit complementary features of both liposomes and polymeric nanoparticles (Hadinoto et al. 2013). Despite their complex structure, they are easily prepared in one-step method by simultaneous self-assembly of the lipid and polymer resulting into better and higher production. Recently, Jiang et al. (2014) developed gel-liposome hybrid nanoparticles for anticancer drug-delivery systems and performed efficient drug delivery with increased fold than that of individual loaded with the drug (Jiang et al. 2014). In another study, it was found that β-D-galactosidase-responsive hybrid SiNPs capped with different saccharide derivatives

could open the gate and release cargoes when exposed to the specific enzymes upon uptake by tumor cells (Bernardos et al. 2010). Therefore, the hybrid nanoparticles can be exploited extensively as the promising platforms for cancer therapy. The current state of development for the hybrid nanoparticle preparations and applications are required to bring them closer to its clinical realization.

6.3 Enzyme-Responsive Nanocarriers in Effective Drug Release for Biomedical Applications

The inherent enzymes play a crucial role at the microenvironment of the target tissues for controlled and targeted drug release for effective therapy of disease. In this section, several approaches that exploit the biocatalytic action of an enzyme for drug delivery, diagnostics, and therapies are summarized. Most of the enzymes interact with the nanocarriers for catalysis in the host system. The most widely used enzymes for drug delivery are hydrolase class of enzymes. These hydrolytic enzymes include proteases, lipases, glycosidases, etc. (Hu et al. 2014). As most of the utilized biocompatible nanoassembles have attachment of bioactive moieties through cleavable units, the nonspecific hydrolases of host can cleave their bonds of integrity through which drug molecules are anchored and results into successful, effective, and controlled drug delivery. Such materials are frequently known as hydrolase-responsive nanomaterials (Mura et al. 2013). Besides, some nanomaterials are responsive to oxidoreductase class of enzymes. The deployment of oxidoreductases is still in a proof-of-concept stage, and some pioneering examples on their utilization for drug delivery are enlisted in Table 6.1.

Enzyme-activated drug carriers expose the drug molecules to the specific targeted tissues and then subsequently internalize into specific cells. The site-specific enzymatic cleavage leads in the release of drugs from different nanocarriers. Drugs can be loaded into nanomaterials through covalent attachment (or physical encapsulation), involving a crosslinked matrix, a self-assembled system, and a caged porous structure (Mura et al. 2013; Hu et al. 2014). Various designs of enzyme-responsive nanomaterials for controlled and effective drug delivery are summarized in Fig. 6.1.

6.3.1 Protease-Responsive Nanocarriers

The expression and activity of specific protease in the diseased tissue is characterized by imbalances in their over- and underexpression of their genes. Protease can be utilized for selective activation of advanced drug delivery platforms. They are known to be involved in many physiological processes such as wound therapy, tissue remodeling, cancer metastasis, etc. (Hu et al. 2014). For instance, peptide-conjugated polymer nanoparticles were synthesized that could overexpress and

Table 6.1 Enzyme responsive nanoplatforms with potential therapeutic applications

Type of enzyme	Responsive enzyme (E.C. number)	Nanomaterial used for immobilization	Therapeutic application	References
Proteolytic enzymes	α-Chymotrypsin (E.C. 3.4.21.1)	Single-enzyme nanoparticles (SEN)	Enzyme responsive drug delivery and biosensing application	Kim and Grate (2003)
	Cathepsin B (E.C. 3.4.22.1)	Polymeric nanoparticles (HPMA)	Intracellular drug delivery	Vicent and Pérez-Payá (2006)
			Extracellular drug delivery	Satchi-Fainaro et al. (2003)
	Cancer associated proteases (CAPs) (3.4._)	Polymer-stabilized liposomes	Targeted drug delivery	Basel et al. (2011)
	Caspase 1 thrombin collagenase chymotrypsin (E.C. 3.4._)	Semiconductor nanoparticles (quantum dots)	Biosensing via FRET	Medintz et al. (2006)
	Matrix metalloproteinases (MMPs) (E.C. 3.4.24._)	Mesoporous silica nanoparticles (MSNPs)	Enzyme responsive drug delivery system	Liu et al. (2015)
	Prostate-specific antigen (PSA) (E.C. 3.4.21.77)	Gold nanoparticles	Prostate cancer diagnosis	Laromaine et al. (2007)
	Papain (E.C. 3.4.4.10)	Carbon nanotubes, CLEA, porous-CLEAs (cross-linked enzyme aggregates)	Enzymatic hydrolysis of macromolecules	Wang et al. (2011)
	Pepsin (E.C. 3.4.4.1)	Polyurethane microsphere/AuNP	Enzyme responsive drug delivery and Immunosensing applications	Phadtare et al. (2003)
	Trypsin (E.C. 3.4.4.4)	Single-enzyme nanoparticles (SEN)	Enzyme responsive drug delivery and biosensing application	Kim and Grate (2003)

(continued)

Table 6.1 (continued)

Type of enzyme	Responsive enzyme (E.C. number)	Nanomaterial used for immobilization	Therapeutic application	References
Lipolytic enzymes	Acetylcholine esterase (E.C. 3.1.1.7)	Polymer nanoparticles	Highly sensitive and microdiagnostic system	Konno et al. (2004)
	Carboxylesterase (E.C. 3.1.1.1)	Silica nanosystems	Biosensing applications and chiral resolution reactions	Edwards et al. (2011)
	Lipase (E.C. 3.1.1.3)	Supermagnetic nanoparticles	Chiral resolution reactions	Gardimalla et al. (2005)
	Phospholipase A_2 (3.1.1.4)	Polymer-stabilized	Synergistic drug delivery	Andresen et al. (2004)
		Liposomes	Phospholipase sensor	Aili et al. (2011)
Other hydrolases	Alkaline phosphatase (ALP) (E.C. 3.1.3.1)	Modified silica coatings	Surgical implant functionalization	Ehlert et al. (2010)
	α-Amylase (E.C. 3.2.1.1)	Polymeric nanoparticles (dextran)	Targeted drug delivery	Ferguson et al. (2009)
		Hybrid nanoparticles	Proteomic analysis, antifouling, biofuel cells, and tissue engineering	Wang et al. (2013)
		Silica nanosystems	Biochemical diagnostics	Bellino et al. (2010)
		Halloysite nanotubes, zeolites, carboxymethyl-tamarind-gum-silica nanohybrids	Food intolerance; improvement of fat digestibility and enhancement of growth performance	Singh and Kumar (2011)
	Arginase (E.C. 3.5.3.1)	Gold nanoparticles	Fibrin clot	Lee et al. (2012)
	Lysozyme (E.C. 3.2.1.17)	Gold nanorods; silica-coated magnetic nanoparticles	Fabrication of bioactive and biocompatible nanoparticle	Yang et al. (2007)
	Urease (3.5.1.5)	Gold nanoparticles	ELISA	de la Rica et al. (2012)

(continued)

Table 6.1 (continued)

Type of enzyme	Responsive enzyme (E.C. number)	Nanomaterial used for immobilization	Therapeutic application	References
Oxidoreductases	Catalase (E.C. 1.11.1.6)	Bentonite and sepiolite nanoparticles, starch-based polymers, metal-chelated affinity cryogels	Biosensing and biomedical applications	Cengiz et al. (2012)
	Choline oxidase (E.C. 1.1.3.17)	Polymer nanoparticles	Highly sensitive and microdiagnostic system	Konno et al. (2004)
	Glucose oxidase (E.C. 1.1.3.4)	Liposomes	Drug delivery triggered by glucose	Napoli et al. (2004)
	Monooxygenase (E.C. 1.14.14.1)	Mesoporous materials	Targeted drug delivery	Weber et al. (2010)
	Peroxidase (E.C. 1.11.1._)	Gold nanoparticles	ELISA	de la Rica et al. (2012)

activate the gene in response to protein kinase or protease activity (Kang et al. 2008). Correspondingly, Law et al. (2006) developed self-assembled peptide nanocarriers which could release therapeutic drugs and degraded peptide fragments upon interaction with proteases associated with infection (Law et al. 2006). Another class of proteases which are often used for controlled drug delivery are known as matrix metalloproteinases (MMPs). MMPs are proteolytic enzymes and have long been associated with cancer and other degenerative diseases (Hu et al. 2014). Their mechanism of action is protein degradation leading to the regulation of various cell behaviors pertaining to diseased physiology. In diseased condition, they are overexpressed in comparison to normal physiological conditions (Nguyen et al. 2015). For example, Jiang et al. (2014) developed a proteolytically activated cell-penetrating peptide-modified nanoparticle drug delivery system triggered by MMP-2 and MMP-9 for cancer therapy. Correspondingly, the overexpressed cancer-associated MMP-2 in tumor tissues catalyzes proteolysis which have made protease-activated effective drug delivery platform for tumor treatment (Jiang et al. 2014). Proteases thus serve as ideal biomolecules for targeted drug delivery in different cell compartments.

6.3.2 Lipolytic Enzyme-Responsive Nanocarriers

Upregulation of lipolytic enzymes, especially phospholipase, has been a pathological indicator for various diseases such as cancer, myocardial infarction, neurodegeneration disorders, wound healing, inflammation, and infectious diseases (Gardimalla et al. 2005; Aili et al. 2011). Phospholipase A_2 (PLA_2) is one such

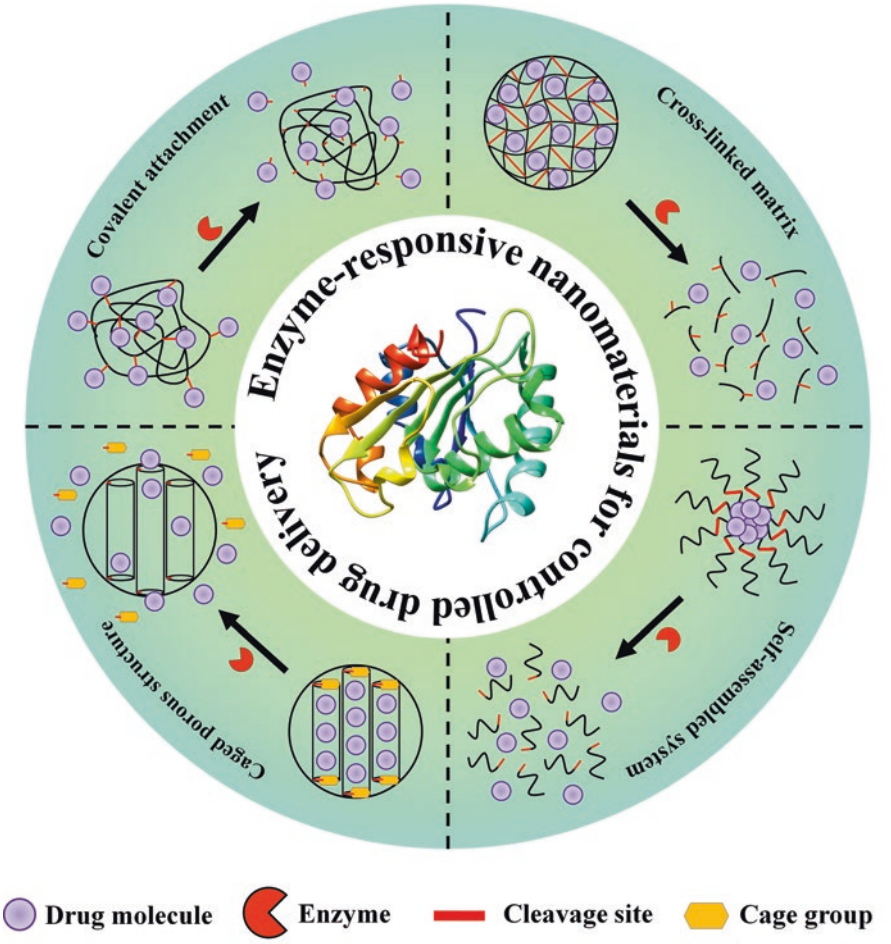

Fig. 6.1 Design of enzyme responsive nanoparticles for efficient drug release

enzyme that is upregulated in cancer tumors. It is observed that in prostate cancers, PLA_2 is expressed at levels greater than disease-free paired controls (Yu et al. 2012). The upregulation of secreted PLA_2 in tumor microenvironment mediates carcinogenesis by (i) release of arachidonic acid producing carcinogenic metabolites, and (ii) release of lysophospholipids, including lysophosphatidic acids inducing cell growth (Yu et al. 2012). This upregulation of PLA_2 enzymes in the microenvironment of tumor is being actively explored for responsive drug release in the tumors (Aili et al. 2011). Further, Andresen et al. (2004) synthesized an enzymatically activated liposome drug delivery system which was involved in masking antitumor ether lipids (AELs) as prodrugs. Results showed that PLA_2-activated proAELs and converted water-soluble prodrugs to chemotherapeutic agents (Andresen et al. 2004). Thus, the generated lipid hydrolytic products after enzyme responsive can

perturb the membranes and enhance the cellular uptake of drugs encapsulated in the liposomes.

6.3.3 Other Hydrolase-Responsive Nanocarriers

The other class of hydrolases including glycosidases, phosphatases, ureases, amidase, etc. have a significant role in activation and effective and controlled drug release at the site of infection (Singh and Kumar 2011; Lee et al. 2012; Wang et al. 2013). Glycosidases (such as α-amylases) catalyze the hydrolysis of carbohydrates to yield small sugar moieties, and thus, they are useful biocatalysts for triggering the target-specific drug delivery when secreted in microenvironment of target tissue (Ferguson et al. 2009). It has been revealed that the enzyme α-amylase is overexpressed up to 85-fold in the tumor tissues, and consequently it should be possible to design polysaccharide-based nanocarriers to release anti-cancer drugs specific to tumors. Further, to prove this concept, enzyme PLA_2 as drug was covalently linked to dextran (R-1,4 poly(D-glucose)) via succinoylation (Ferguson et al. 2009). Earlier, it was reported that the PLA_2 was used for treatment of bladder, breast, cervix, and lung cancers in clinical phase I, where more than 50% of patients treated with the drug exhibited neurotoxicity (Wang et al. 2013). However, when conjugated to sugar, PLA_2 activity was shielded, and systemic toxicity was not observed (Wang et al. 2013). Correspondingly, PLA_2 was released at desired concentrations when α-amylase was added at optimal required concentrations and exhibits the functionalization of the nanocarrier. Similarly, Bernardos et al. (2010) reported that lactose or starch derivatives grafted mesoporous silica nanoparticles (MSNPs) can be employed for enzymatically elicited drug release (Bernardos et al. 2010). On the other hand, Harnoy et al. (2014) employed enzyme penicillin G amidase to cleave the end groups of phenyl acetamide that could disassemble the synthesized micelles for controlled drug release at the diseased tissue (Harnoy et al. 2014). Thus, hydrolases are interesting biomarkers helpful in designing advanced drug delivery systems with enhanced features.

6.3.4 Oxidoreductase-Responsive Nanocarriers

Oxidoreductases are promising target for drug delivery systems where various diseases such as cancer, diabetes, neurodegenerative diseases, etc. create oxidative environments (Napoli et al. 2004). The glucose oxidase (GOx), catalase, and peroxidase are extensively exploited for enzyme-responsive controlled drug release systems (Napoli et al. 2004). Gu et al. (2009) applied chitosan-coated nanoparticles with a solid core of insulin and enzymes (GOx and catalase) that was responsive to blood–sugar levels for closed-loop insulin delivery in the therapy of diabetes. At hyperglycemic state, glucose was converted into gluconic acid by GOx which

resulted in dissociation of porous nanonetwork and subsequently release of insulin to treat diabetes (Gu et al. 2009). Other enzymes like azoreductase has established widespread attention in the treatment of colon diseases as they are secreted by gut microflora. Rao and Khan (2013) synthesized azoreductase responsive nanoplatforms by copolymerization of drug and azobenzene-linked poly(ethylene glycol)-b-poly(styrene). As the system was azoreductase sensitive and selective, it has the potential for treatment of colon diseases (Rao and Khan 2013). Overall, oxidoreductase responsive nanocarriers open up new avenues toward the development of favorable drug delivery systems.

6.4 Salient Features of Enzyme Immobilized Nanocarriers for Biomedical Applications

To reduce the side effect of engineered nanoparticles, it is important to study associated factors for successful drug (as enzyme) delivery. The vital goals for optimized therapeutic drug delivery that are provided by medical pharmacology include the right nanoparticle design, right drug, right administration route, and right drug dosage at the right time to the right patient (Li 2014). The factors involved in efficient delivery that includes drug chemical composition, size of nanoassembly, surface modification, biocompatibility, route of drug administration, sufficient enzyme load to their site-specific action, targeted therapy for subcellular localization, and duration of drug delivery as illustrated in Fig. 6.2. These optimally designed nanoassembly which will not elicit immune system have been investigated for treatment of various pathological conditions.

6.4.1 Size of Nanoassembly

Nanosize and their size distribution are the most important characteristics of enzyme-loaded nanocarriers. It determines the biological fate, biodistribution, biocompatibility, toxicity, enzyme load, and targeting ability of the nanocarrier. The methods used for determination of these nanoassembly size is by photon-correlation spectroscopy (PCS) or dynamic light scattering (DLS), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Out of them, most routinely used method of size determination is by PCS or DLS. Photon-correlation spectroscopy requires the viscosity of the medium to be known and determines the diameter of the particle by Brownian motion and light scattering properties (Gorbenko and Trusova 2011). The results obtained by photon-correlation spectroscopy are usually verified by SEM or TEM. The advantages of using biocompatible nanocarriers (nanoparticles) over microcarriers (microparticles) for therapeutics are that (a) it can cross the blood–brain barrier easily through endothelium tight

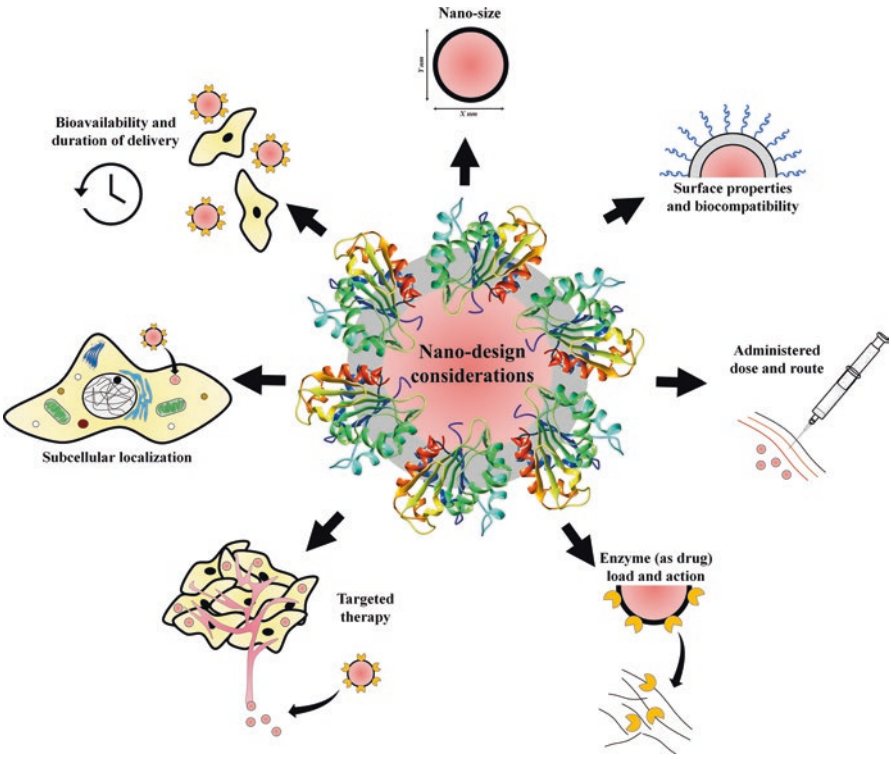

Fig. 6.2 Nanodesign considerations for successful enzyme (or drug) delivery

junctions (Saraiva et al. 2016), (b) nanoassemblies have a many fold greater uptake rate than that of microparticles (Desai et al. 1997), (c) nanocarriers have greater penetrability throughout the intestinal submucosal layers in comparison to microparticles that are predominantly localized in the epithelial lining (Redhead et al. 2001), and (d) nanoparticles have a larger surface area to volume ratio leading to faster immobilized enzyme activity as well as stability in contrast to microparticles, that have large cores and allows more enzymes to be encapsulated per particle giving slower enzyme activity (Redhead et al. 2001). Therefore, control of particle size helps in tuning enzyme action rates.

6.4.2 Surface Modifications of Nanomaterials for Enhanced Biocompatibility

Although most of the researchers have acquired detailed understanding about the behavior of nanomaterial surfaces, still the surface chemistry of nanoparticles should explore and control surface parameters of nanoparticles such as charge,

hydrophobicity, porosity, etc. by various nanotechnological approaches for biomedical applications (Aggarwal et al. 2009). The nature of surface interface is dependent upon the biological applications that impacts nanoparticle properties. The surface charge of nanoparticle plays a significant role in determining its interaction with cell membranes for intracellular drug distribution (Calvo et al. 1997). The positively charged nanoparticles can penetrate deep into cell membranes because of negatively charged cell surface, whereas negatively charged nanoparticles does not at all enter into the cell that could help in engineering of nanoparticles for therapeutic drug delivery. The zeta potential of a nanoparticle is commonly used to characterize the surface charge property of nanoparticles (Couvreur et al. 2002). The aggregation of nanoparticle is prevented when zeta potential is above ±30 mV due to their surface charges and regarded as stable in suspension. It is known that the nonmodified hydrophobic nanoparticles can easily be recognized by phagocytes and blood components (e.g., opsonins) upon administration and then cleared from the host circulatory system (Singh and Lillard 2009). Thus, nanoparticles hydrophilicity can be attained by coating with hydrophilic polymers/surfactants such as polyethylene glycol (PEG), polyethylene oxide, polyoxamer, poloxamine, Tween 80, etc. (Singh and Lillard 2009). Olivier (2005) shows that coated nanoparticle with PEG prevents opsonization and the administered nanoparticle remained active after reaching the target tissues. Their result also showed the brush-like configurations of PEG on nanoparticle surface reduce phagocytosis and complement activation, whereas mushroom-like structures are more prone to complement activation and favored phagocytosis (Olivier 2005). The successful surface modifications of nanocarriers lead to host mimetic functionalization that improves the biocompatibility and specific biological interactions.

6.4.3 Dosage and Administered Route for Therapeutic Nanocarriers

The main goal for both administered route and immobilized drug (or enzyme) dosage are that they uptake by specific target cells, and retain the enhanced delivery, reduce the toxic effects of free drug at non-target organs, and have therapeutic efficacy (Singh and Lillard 2009). Understanding the existing methods for drug administration and appropriate route for their application completely depends on their target tissues or sites of their distribution. Various bioactive cargos such as drugs, enzymes, antibodies, whole cells, etc. are administered for their biological activity evaluation. Thus, engineering nanocarriers that are target-specific and have longevity in circulation is required (Moghimi et al. 2001). Nowadays, drug-loaded nanocarriers are coated with biodegradable polymers causing drug release post degradation after long travel at specific tissues (Olivier 2005). For achieving successful target-specific delivery, nanocarrier design should consider environmental pH, concentration, viscosity, toxicity levels, etc. All administration routes have both

Table 6.2 Advantages and disadvantages of route of administrations for targeted delivery

Route of administration	Advantages	Disadvantages
Oral	Noninvasive means of nanoparticle delivery	First pass metabolism in the liver potentially hepatotoxic; potential for translocation into systemic circulation; requires intact intestinal mucosa for nanoparticle uptake
Parenteral	Preferred for rapid absorption, useful when patient is unconscious, noncooperative, or unable to take drug by an enteral route	Difficult to remove; dose is toxic; risks involved with invading the body with a needle
Pulmonary	Noninvasive means of nanoparticle delivery; large surface area; local action; avoidance of first pass metabolism in the liver	Local toxicity; potential for translocation into systemic circulation
Transdermal (local)	Noninvasive means of nanoparticle delivery; large surface area; local action	Local irritation; potential for translocation into systemic circulation
Intravenous	Systemic delivery of nanoparticles; systemic action	First pass metabolism in the liver potentially hepatotoxic; systemic toxicity
Intrathecal	Useful in spinal anesthesia, chemotherapy or pain management applications	Difficult dose calculation/difficult technique; direct access to CNS creates danger

advantages and disadvantages for targeted delivery like the bioavailability, absorption, metabolism, etc. (Singh and Lillard 2009). Some of the advantages and disadvantages for targeted delivery of some route of administrations are enlisted in Table 6.2. A detailed conceptual understanding of dosage and administered route is required for the development and application of safe nanomaterials in drug delivery.

6.4.4 Loading of Enzyme onto the Nanocarriers

A good, smart and successful nanocarrier delivery system should have a high enzyme (as drug) loading capacity, high immobilization efficiency, and yield for their administration into the host system (Meryam Sardar 2015). The loading of enzymes/drugs on the nanocarriers can be carried out by adding drug molecules at the time of their synthesis or by adsorbing the drug molecules after the formation of nanoparticles. Thus, the enzyme (as drug) loading can be achieved via physical adsorption, covalent attachment, crosslinking, entrapment, or self-assembly and

with nanomaterial (Fig. 6.1). The major factors affecting therapeutic molecules loading are molecular weight of enzyme, matrix composition, functional groups of the matrix, enzyme–polymer interactions, enzyme–polymer solubility and incubation time for assembling (Panyam et al. 2003). In addition, Calvo et al. (1997) reported that the macromolecules such as drugs or enzyme encapsulated are efficiently loaded in nanoparticles at or near their isoelectric point as drug and matrix materials interacted through ionic bonds (Calvo et al. 1997). As a result of the increased and efficient enzyme/drug loading on the carrier, their sustainable release rate at the site of action will be regulated.

6.4.5 Tissue Specific-Targeted Delivery of Nanocarriers

Currently, research focuses toward "smart drug" for therapeutic efficacy that is also referred to as tissue-specific targeted drug delivery. There are two broad areas of targeted drug delivery: (i) systemic targeting and (ii) intracellular targeting (Panyam et al. 2003). The classification of the current targeted drug delivery processes are represented in Fig. 6.3A. Systemic targeting is classified into ligand–receptor mediated and locally activated drug delivery that is based on blood circulation and extravasation (Bae and Park 2011). Further, locally activated drug delivery can occur either through self-triggered drug release by the site specific signal such as presence of specific enzymes or pH changes or occur by externally activating drug release by physical factors like light, temperature, magnetic field, and ultrasound. On the other side, intracellular targeting can be achieved through intracellular trafficking of the drug carriers to specific locations within targeted cells (Bae and Park 2011). For intracellular targeting, the drug delivery strategies have been divided into two categories: (a) passive and (b) active. Passive drug targeting is frequently known for enhanced permeation and retention (EPR) effect (Maeda 2010). It occurs through accumulation of drug at the site of tumors with having leaky vasculatures. The EPR effect is seen only when the drug nanocarriers are intravenously administered to the host. Thus, it shows side effect as majority of the drug might accumulate in other organs (Maeda 2010). On the other hand, active drug targeting describes the specific interactions between ligand and receptor that results into subcellular localization of drug/drug carrier to the target cells, which occurs only after blood circulation and extravasation (Bae and Park 2011). This is why PEGylation improving the EPR effect is expected to enhance delivery to the tumor site by increasing blood circulation time (Bae and Park 2011). Drug targeting strategies through active and passive drug delivery by the EPR effect are illustrated in Fig. 6.3B. Targeted delivery of nanocarriers lead to improved pharmacokinetic properties thus increasing the safety and maximizing the therapeutic effects.

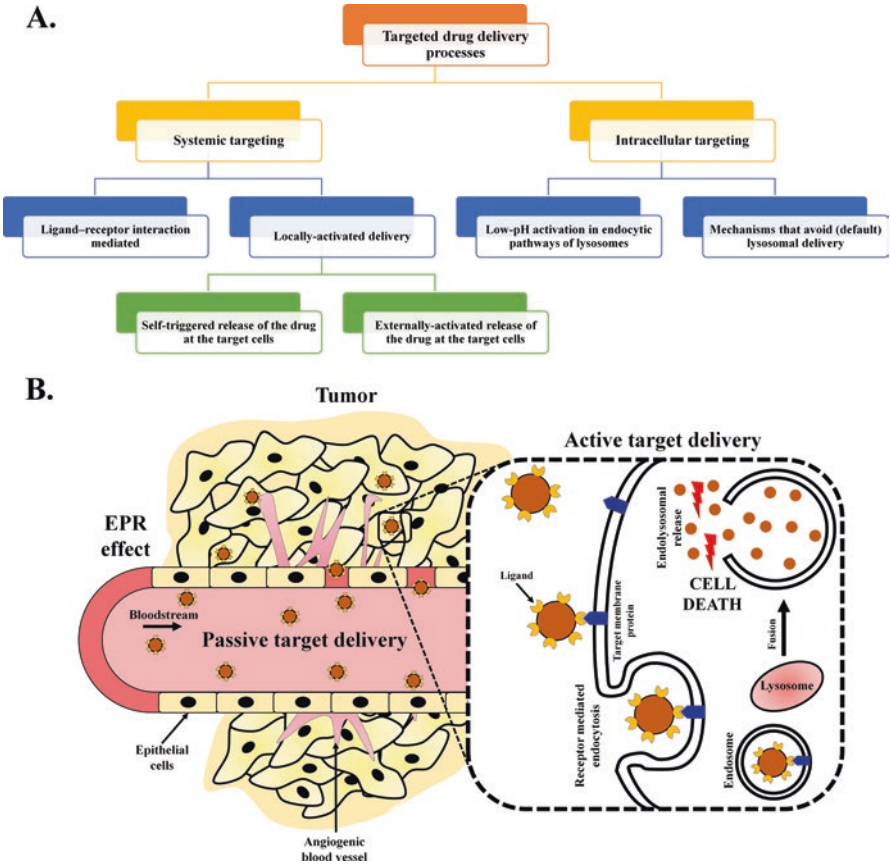

Fig. 6.3 Strategies for targeting of nanocarriers. (**A**). Types of targeted drug delivery processes; (**B**). Mechanism of active and passive drug delivery

6.4.6 Biodistribution and Subcellular Localization for Therapeutic Efficacy

The intrinsic physiochemical properties of drug loaded nanocarriers vary for different physiological sites which aim at successful biodistribution, subcellular localization, and then targeted molecular action of nanocarriers after cellular uptake (van Vlerken et al. 2007). The effective parameters for required biodistribution and subcellular localization of functionalized nanocarriers are also affected by their morphology, surface charge, and chemical composition of the nanomaterials (Olivier 2005). Consequently, drug-loaded nanocarriers are taken up through specific endocytic and trafficking pathways that modulate subcellular drug distribution by altering enzyme levels and cellular signaling pathways that finally results into increase in induction of disease therapy. Thus, the fate of drug-loaded nanocarriers for

subcellular localization is appropriate for their therapeutic efficacy and biosafety (Tansi et al. 2017). Moreover, functionalization of nanocarriers is significant for successful and targeted biodistribution in organisms (Aggarwal et al. 2009). It inhibits the nanoparticles binding to different blood proteins, including opsonins which evoke nanoparticle opsonization and phagocytosis in an organism. For instance, functionalization through PEGylation suppresses phagocytosis of nanoparticles and leads to enhanced systemic circulation (Chaudhari et al. 2012). Therefore, designing and engineering of nanoparticles facilitate required effective biodistribution and subcellular localization.

6.4.7 Bioavailability and Duration of Drug Delivery for Successful Therapeutics

Bioavailability is one of the key factors for successful drug delivery in which the natural barriers encountered is the determining factor when administered. To improve this, extensive research is being carried out to overcome these natural barriers through engineering nanocarriers to transport drugs across biological barriers at the targeted site (Singh 2015). Thus, for successful therapeutics, the continuous release of drug from the nanocarrier should extend over a period of time after their administration in the host. The prolonged therapeutic effect of drug depends upon controlled drug release, sustained and extended period of action, and time of release dosage (Singh and Lillard 2009). The bioavailability of a drug to the targeted pathological site also depends on various physicochemical factors such as its water solubility, dissolution rate, metabolic rate, duration of delivery, membrane permeable and susceptibility to efflux mechanisms, the mode of exposure (intravenous, dermal, inhalation, and oral), and its fate (absorption, distribution, metabolism, and excretion) (Dmochowski and Staskin 2002). The bioavailability of the nanodrug carriers should alter the effect of their degrading pathways and establish the drug level in the host. These attributes are integrated during the drug carrier designing to maximize the benefits of designed drugs. Nowadays, most of drug candidates are insoluble in water, and it limits the in vivo bioavailability which is an important challenge that opens new avenues for the scientific society (Dmochowski and Staskin 2002).

6.5 Immobilized Enzymes as Drug for Therapeutic Delivery

The immobilization of therapeutic enzymes as drug delivery has been classified according to the type of nanomaterials, their chemical composition, its structural properties, and the route of administrations of the nanocarrier are decided accordingly for various biomedical applications. For most of the clinical applications, the

new smart delivery of nanoenzymes depends upon choosing of drug for therapeutics like cancer, antithrombotic therapy, wound healing, alcohol intoxication, gastrointestinal diseases, etc. (Bosio et al. 2015). The various immobilized enzymes for biomedical therapeutic applications are enlisted in Table 6.3. To cite an example, in the therapy of cardiovascular arrest, multiple blood proteases (such as tissue plasminogen activator and urokinase plasminogen activator) in their nanoimmobilized form find potential application for antithrombosis therapy to treat blood clots by degradation of insoluble fibrin into soluble fibrin (Park et al. 2001; Piras et al. 2008). Besides, microbial proteases (like *Bacillus subtilis* nattokinase and *Candida albicans* lumbrokinase) are commonly used for different immobilization strategies to facilitate antithrombosis therapy as they have higher efficacy for fibrinogen cleavage (Ren et al. 2010). Although impediments still exist in the use of immobilized enzymes as drug, the potentiality and the versatility of these novel catalysts put forward a bright future for an emerging drug therapy approach.

6.5.1 Applications of Immobilized Enzymes in Treatment of Diseases

During the last decades, immobilized enzymes have been studied vigorously for its potential in biomedical applications for remedy of multiple diseases without any immunological effect. Immobilized enzymes are used in therapy of various illnesses like cancer and wound healing, in inborn metabolic defects, for alcohol intoxication, etc. The nanocarrier immobilized enzyme forms are more prone to combat the diseases by scavenging accumulated toxic metabolic products (Bosio et al. 2015).

Since the last three decades, immobilization of various enzymes has been employed for potential applications of cancers. For instance, earlier in 1997, Storm et al. employed immunoliposomes delivering β-glucuronidase for treatment of cancer by activation of anticancer prodrugs (Storm et al. 1997). Löhr et al. (2001) used microencapsuled cytochrome P450 enzymes using sodium cellulose sulfate and polydialyldimethylammonium chloride that were involved in the removal of carcinogenic compounds from the body to convert ifosfamide to its cytotoxic metabolite (Löhr et al. 2001). Correspondingly, PEG-conjugated immobilized adenosine deaminase was used for anticancer therapy, especially, for melanoma and hepatocarcinoma (Kim et al. 2009). On the other hand, asparaginase is an important enzyme used effectively and potentially for the acute lymphoblastic leukemia treatment by hydrolyzing the L-asparagine to aspartic acid and ammonia that causes targeted cell death. It is coimmobilized with drugs that are important for therapeutics of cancer and modified by PEGylation for enhanced efficiency of the drug delivery for the treatment of acute lymphoblastic leukemia (Wang et al. 2013). In other studies, asparaginase is immobilized in various polymeric nanomaterial such as poly(lactide-co-glycolide), activated agarose-glutaraldehyde supports, liposome, etc. for clinical testing in the in vivo systems (Cruz et al. 1993; Manuela Gaspar

Table 6.3 Nanoimmobilized enzymes for therapeutic and their route of administrations

Therapeutic enzymes (E.C. numbers)	Disease therapy	Immobilization nanomaterial	References
Parenteral administration of nanocarrier			
Alcohol oxidase (E.C. 1.1.3.7) and catalase (E.C. 1.11.1.6)	Alcohol intoxication and Alcohol prophylaxis	DNA-directed assembly with AuNPs	Liu et al. (2013)
Asparaginase (E.C. 3.5.1.1)	Acute lymphocytic leukemia	Poly(lactide-co-glycolide) nanoparticles	Manuela Gaspar et al. (1998)
		Liposome formulations of simplified dehydration-rehydration vesicles or extruded vesicles	Cruz et al. (1993)
		Polyethylene glycol conjugated enzymes	Liu et al. (2016)
		Multi-subunit covalent immobilization of the enzyme onto activated agarose-glutaraldehyde supports	Balcao et al. (2001)
Cytochrome P450 (cells producing the enzymes) (E.C. 1.14.14.1)	Cancer (to convert ifosfamide to its cytotoxic metabolite)	Microencapsulation of sodium cellulose sulfate and poly(dialyldimethylammonium) chloride	Löhr et al. (2001)
Glucose-6-phosphate-dehydrogenase (E.C. 1.1.1.49)	Jaundice	Silica-based matrix	Cumana et al. (2013)
Pepsin (E.C. 3.4.23.1), chymotrypsin (E.C. 3.4.21.1), trypsin (E.C. 3.4.21.4)	Gastrointestinal diseases and fat malab-sorption (Enzyme replacement therapy)	Enzymes injected into Creon® Minimicrospheres	Santini et al. (2000) and Patchell et al. (2002)
Serine endopeptidase (lumbrokinase, E.C. 3.4.21) and subtilisin (nattokinase, E.C.3.4.21.62)	Antithrombotic therapy	Magnetic nanoparticles	Ren et al. (2010)
Streptokinase (E.C. 3.4.99.22)	Thrombolytic therapy	Enzyme drug to be administered in an inactive (i.e., prodrug) form and then released at the target site in an active form using protamine as the triggering agent	Liang et al. (2000)
Streptokinase (E.C. 3.4.99.22)	Antithrombotic therapy	RGD-(Ar–Gly–Asp)-peptide-conjugated liposomes (RGD Lips), magnetic nanoparticles	Walde and Ichikawa (2001)

(continued)

Table 6.3 (continued)

Therapeutic enzymes (E.C. numbers)	Disease therapy	Immobilization nanomaterial	References
Tyrosinase (E.C. 1.14.18.1)	Cancer (treatment of melanoma cancer skin)	Polylactic-acid nanocapsules, polyethyleneglycol–polylactic acid nanocapsules	Wang and Chang (2012)
t-Plasminogen activator (E.C.3.4.21.68,)	Antithrombotic therapy	Magnetic nanoparticles, PEGylated liposomes, poly-(lactide-co-glycolide)/chitosan nanoparticles, polystyrene latex nanoparticles	Park et al. (2001)
u-Plasminogen activator or urokinase (E.C. 3.4.21.73)	Antithrombotic therapy	2-methoxyethanol hemiesters of poly(maleic anhydride-alt-butyl, vinyl ether) nanoparticles	Piras et al. (2008)
Local administration of nanocarrier			
Adenosine deaminase (E.C. 3.5.4.4)	Cancer (melanoma and hepatocarcinoma)	Polyethylene glycol conjugated enzymes	Ensor et al. (2002)
Alkaline phosphatase (E.C. 3.1.3.1)	Hypophosphatasia (Increased mineralization and bone regeneration)	Microporous nanofibrous fibrin scaffolds	Osathanon et al. (2009)
β-Glucuronidase (E.C. 3.2.1.31)	Cancer (treatment by activation of anticancer prodrugs)	Immunoliposomes bearing enzymes (immunoenzymosomes) for site-specific activation of anticancer prodrugs	Storm et al. (1997)
Brachyurin (collagenase, E.C. 3.4.21.32)	Skin ulcers	Hyaluronan microparticles	Lee et al. (2001)
Catalase (E.C. 1.11.1.6)	Wound healing	Sugar–ester vesicles	Thiem and Goślińska (2004)
Heparin lyase (E.C. 3.2.1.19)	Wound healing	Gold, titanium and zinc oxide nanoparticles	Behera et al. (2012)
Keratinase (E.C. 3.4.4.25)	Debridement of necrotic tissue	Polyvinyl-alcohol cryogels	Martínez et al. (2013)
Lysozyme (E.C. 3.2.1.17)	Wound healing and other antimicrobial therapies	Titania nanotubes, carbon nanotubes, mesoporous silica rods, nanofiber	Huang et al. (2013)
Matrix metalloproteinases (MMPs) (E.C. 3.4.24._)	Cancer (treatment by enzyme responsive drug delivery system)	Mesoporous silica nanoparticles	Liu et al. (2015)
Papain (E.C. 3.4.4.10)	Wound healing	Niosomes and nanospheres	Manosroi et al. (2013)
Trypsin (E.C. 3.4.4.4)	Wound healing	Cotton yarn	Nikolic et al. (2010)

(continued)

Table 6.3 (continued)

Therapeutic enzymes (E.C. numbers)	Disease therapy	Immobilization nanomaterial	References
Oral administration of nanocarrier			
β-Galactosidase (E.C. 3.2.1.23)	Lactose intolerance	Magnetic nanoparticles	Corchero et al. (2012)
Bilirubin oxidase (E.C. 1.3.3.5)	Neonatal jaundice	Immobilized rat-serum albumin	Soltys et al. (1992)
Chymotrypsin (E.C. 3.4.21.1)	Pancreatic insufficiency	Magnetic nanoparticles; electrospinning nanofibers	Medeiros et al. (2011)
Glucose oxidase-peroxidase (E.C. 1.1.3.4)	Oral infections	Encapsulation of enzymes glucose oxidase and in combination with horse radish peroxidase by both extrusion and reverse-phase evaporation	Hill et al. (1997)
Phenylalanine ammonia lyase (E.C. 4.3.1.24)	Phenylketonuria	Immobilized in semipermeable microcapsules for enzyme replacement in phenylketonuria	Bourget and Chang (1985)
		Cross-linked enzyme aggregates	Cui et al. (2012)
Phosphotriesterase (E.C. 3.1.8.1)	Intoxication by organophosphates	Enzymes encapsulated within sterically stabilized liposomes	Petrikovics et al. (1999)
Prolidase (E.C. 3.4.13.9)	Prolidase deficiency (enzyme replacement therapy)	Enzyme loaded into biodegradable poly(d,l-lactide-co-glycolide) microspheres	Genta et al. (2001)
Urease (E.C. 3.5.1.5)	Removal of urea in kidney failure	Orally ingested microencapsulated urease to remove urea in kidney failure	Wolfe and Chang (1987)
Xanthine oxidase (E.C. 1.1.3.22)	Lesch–Nyhan	Microcarrier	Palmour et al. (1989)
		Concanavalin A layered calcium alginate–starch beads; positively charged trimethylammonium-functionalized mixed-monolayer protected clusters	Haider and Husain (2008)
Intravenous administration of nanocarrier			
Alcohol dehydrogenase (E.C. 1.1.1.1) and acetaldehyde dehydrogenase (E.C. 1.2.1.10)	Alcohol intoxication and Alcohol prophylaxis	Encapsulated into human erythrocytes by electroporation	Lizano et al. (1998)
β-Glucosidase (E.C. 3.2.1.21)	Gaucher's disease	Encapsulation in liposomes	Korablyov et al. (1999)
Intrathecal administration of nanocarrier			
Superoxide dismutase (E.C. 1.15.1.1)	Cerebral ischemia and reperfusion injury	Encapsulation in polybutylcyanoacrylate or poly (lactide-co-glycolide) nanoparticles.	Yun et al. (2013)

et al. 1998; Balcao et al. 2001). In recent times, nanoimmobilized arginase has been considered as an alternative for cancer treatment since it catalyzes the conversion of L-arginine to L-ornithine and urea that hinders the growth of cancer tumors (Hsueh et al. 2012). Stasyuk et al. (2011) developed β-mercaptohexadecanoic acid functionalized AuNPs for immobilization of recombinant human type I arginase that resulted in retaining of total enzyme activity. However, it requires further clinical testing for successful cancer therapy (Stasyuk et al. 2011).

Further, tissue repair and wound healing have achieved enormous development in the current scenario of medical research. A complex of enzymatic reactions are carried out at the site of wound that is of major importance for healing of chronic wound. This will reopen new avenues for the nanobased drug delivery approach using immobilization of enzymes such as trypsin, keratinase, papain, pepsin, catalase, arginase, lysozyme, etc. (Bosio et al. 2015). For example, immobilized trypsin has potential applications in wound healing when immobilized covalently with oxidized cotton yarn by sodium periodate (Nikolic et al. 2010). Correspondingly, lysozyme has antimicrobial therapies as it attacks the cell wall of bacteria and hydrolyzes glycosidic linkage of peptidoglycan present in the cell wall. Lysozyme alone or along with trypsin were immobilized on various nanomatrix such as cellulose, PEG methacrylate, silica devices, carbon nanotubes, etc. that were utilized for implantable devices involving supportive wounds (Nepal et al. 2008). Similarly, Martínez et al. (2013) used keratinase which were entrapped in polyvinyl alcohol–pectin cryogel and coimmobilized with antibiotic enrofloxacin for promoting epithelial tissues at the site of wound of skin (Martínez et al. 2013). Further, enzyme catalase is prospective for biomedical applications that prevent oxidative damage by scavenging free radicals produced at the healing site of wound. Catalase was encapsulated in flexible sugar–ester vesicles for in vivo wound healing application, the enzyme was much stable than its free form and retained 95% activity after 90 days (Abdelmajeed 2012). Moreover, the enzymes from plant sources are also employed for healing of wounds. For example, *Carica papaya*-derived papain has potential for removal of dead, necrotic, or infected tissue to improve the wound healing. Manosroi et al. (2013) synthesized gel encapsulated elastic niosomes with immobilization of papain for effective sustained release at the rabbit skin scars (Manosroi et al. 2013).

Another disease where immobilized enzymes find its applications is alcohol intoxication that is a clinical condition leading to psychological disturbances of consciousness. It is a pathological condition in which alcohol enters faster in the bloodstream than that of its metabolization (Bosio et al. 2015). In the body, the consumed ethanol is broken down into nonintoxicating by-products by two enzymes, namely, alcohol-oxidase and catalase. Alcohol oxidase first catalyzes the conversion of ethanol to acetaldehyde and hydrogen peroxide, and then catalase decomposes the later to prevent formation of free radical (Bosio et al. 2015). Recently in 2013, Liu et al. developed novel nanoassemblies of single-stranded DNA, covalent inhibitor of enzymes (such as 4-dimethylamino antipyrine, glucosamine, or lactobionic acid) and lacking enzymes (alcohol-oxidase and catalase) for the alcohol intoxication therapy. Both the enzymes were preconjugated with inhibitor, and then they

were subjected to coencapsulation to produce the scaffolds at specific sequences of DNA that forms nanoassembly of multienzyme system to prevent alcohol intoxication (Liu et al. 2013). Finally, the encapsulation of enzyme–DNA scaffolds by polyacrylamide gels was carried out for enhanced alcohol oxidation rate in ethanol-fed mice (Liu et al. 2013).

In another instance, immobilized enzymes are used for treatment of Gaucher's disease. It is a genetic lipid storage disease which is caused due to deficiency of glucocerebrosidase (membrane-bound lysosomal hydrolase) leading to glucocerebroside deposition in the macrophage-monocyte system at spleen, liver, bones, etc. (Mistry et al. 2017). The substitution of this enzyme in a nanocarrier such as liposome, silica, metallic nanoparticles, etc. is an innovative way of Gaucher disease therapeutics. Thus, Alftrén and Hobley (2013) synthesized activated superparamagnetic magnetite nanoparticles using 3-aminopropyl-triethoxysilane on which glucocerebrosidase were immobilized (Alftrén and Hobley 2013). Further, Alftrén and Hobley (2013) synthesized nonporous iron magnetic particles to immobilize the deficient enzyme through covalent attachment after activated with cyanuric chloride and polyglutaraldehyde. A significant performance was noticed with high activity of immobilized enzyme for the therapy of Gaucher's disease (Alftrén and Hobley 2013). In a nutshell, the technology of immobilized enzymes in therapeutics has evolved and reflected in the ever-broadening applications in treatment of various diseases.

6.6 Challenges and Suggestive Notes

Despite the escalating progress in the design and application of enzyme-responsive and immobilized nanomaterials for therapeutics, there are various challenges that needs to be addressed. Firstly, the main challenge encountered in efficacious drug delivery is optimized designing and adsorption of drugs (or enzymes) on nanocarriers that requires combination of unique nanoassemblies into larger superstructures and tunable functionalities, along with increased stability, solubility, and biocompatibility (De Jong and Borm 2008; Chakravorty et al. 2017). Secondly, target-specific delivery and intracellular localization of nanocarriers are required. Thus, specific interactions between nanoparticles and biological targets must be favored via designing of specific ligands like small molecules, oligosaccharides, peptides, antibodies, and aptamers onto the surface of nanocarriers (Xiao and Farokhzad 2012). Thirdly, release rate of drug at the target site can be tailored by use of enzyme specific nanomaterial for targeted tissue delivery (Singh and Lillard 2009). Fourthly, the difficulty of biological barriers to drug delivery can be overcome through defining the systemic administration route of nanocarriers. This can be achieved by designing multifunctional layers of nanocarriers that can sequentially cross these barriers one at a time. Accordingly, the size, solubility, and pharmacokinetic factors can be modulated depending on the physicochemical properties of the nanocarriers (Li 2014). Fifthly, to gain insight into the immunological responses to nanocarriers,

studying the nanoparticle immunomodulatory effects (such as immunostimulatory and immunosuppressive) is required to understand the physicochemical parameters that define their effects on the immune system so that the nanoparticles can be engineered accordingly (Zolnik et al. 2010). In addition to this, nanocarriers should have improved stability, favorable biodistribution profiles, slower drug release kinetics, lower immunotoxicity, and targeting specific cell populations in order to overcome the toxic effects of nanomedicines (Zolnik et al. 2010). Finally, to maintain drug (or enzyme) concentration at the target site, active drug availability at the site of action for required time and duration should be ensured through optimal delivery system. Taken together, advancement in therapeutic uses for enzymes will open up new vista for biomedical applications of enzyme-responsive and enzyme immobilized nanoplatforms.

6.7 Conclusion and Future Perspectives

Bionanotechnology has great attention in the field of drug delivery applications. A number of unexpected discoveries have been done recently on enzyme-responsive nanocarriers and nanoimmobilized enzyme as drug. A variety of biocompatible nanomaterials are exploited for nanoformulations in treatment of numerous diseases such as cancer, neurodegenerative diseases, diabetes, myocardial infarction, wound healing, inflammation, infectious diseases, etc. It includes protein or polysaccharide-based biopolymers, natural or synthetic or semisynthetic biopolymers and combination of these biopolymers for drug delivery applications. These nanoparticle-based drug or enzyme system can provide improved half-life, high biocompatibility, reduced immunogenicity, and site-specific targeting and overcome the biological barriers to drug delivery. The performance of these nanocarriers has been exploited for the designing of smart drug delivery systems. Such drug delivery systems can be made by polymeric nanoparticles, liposomes, inorganic (such as metal oxide based) nanoparticles, and hybrid nanoparticles for sustained drug release by enzyme responses at the target tissues. Newer methods to obtain innovative technologies for nanocarrier designing have been developed for biomedical applications taking into consideration the key features of immobilized enzyme nanoplatform such as controlled size and shaped nanoparticles, surface modification for biocompatibility, their route of administration, enzyme load for their action, dosage, duration, and site-specific target drug delivery.

Nowadays, research focuses on identifying new smart nanomaterial for engineering them to design biocompatible nanocarriers which can alter its own structure and function in response to the physiological environment. The biocompatible nanoparticle at molecular level therapy needs to be enhanced. Advanced and smart drug (or enzyme as drug) delivery systems has been attained efficaciously in case of cancer. Apart from that, numerous challenges remain to be addressed for other pathologic conditions. For the fabrication and designing of nanoassembly, most of the delivery systems fails to do well in clinical trials. Thus, further research is

required to come up with simple, economical, effective, and accurate preparations with wide applicability for the development of smart delivery systems. Recently, in the field of polymer science with progressive techniques of chemistry, many pioneering approaches are employed to face the difficulties against the upcoming rejection of nanocarriers. Therefore, further investigation is required for biofunctionalization modification of nanocarriers to attain compatibility under physiological conditions. The advancement of next generation advanced nanodesigning of therapeutic carriers should be accorded toward development of compatible nanoassembly by incorporating the cell ligands and receptors, growth factors, antibodies, genes, peptides, etc. to enhance the efficiency and minimize the unwanted effects.

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Chapter 7
Systemic Nanotoxicity and Its Assessment in Animal Models

Vishal Sharma, Bharti Aneja, Vinod Kumar Yata, Dhruba Malakar, and Ashok Kumar Mohanty

Contents

Abstract Nanoparticles (NPs) have found applications in large number of fields which increases the exposure of human to NPs. The potential toxicity of the NPs during the earlier days was not in focus, but during the last decade, much attention has been paid to the potential risk of NP toxicity to human health. In this chapter we summarized biokinetics of NPs and the toxicity of NPs to specific organ systems of the body and different methods used to assess the nanotoxicity using in vivo models. The physicochemical properties of NPs affect their biokinetics and thus toxicity

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to different organ systems in the body. Cationic NPs enhance their cellular uptake compared to neutral or anionic NPs. NPs are absorbed through different routes and are distributed locally or to distant organs by hematopoietic system. NPs may cross the different barriers in the body and cause toxic effects in different organs and even to the developing embryo. NPs smaller than 5 nm can cross the placental barrier and are highly toxic to the developing embryo. NPs of size more than 80 nm are accumulated in different organs and are rarely excreted in feces or urine. Accumulation of the NPs may disturb the homeostasis and result in the toxicity to different levels. NPs are more toxic to the lung, brain, intestine, stomach, and developing embryo. Si and TiO_2 NP absorption through placenta may lead to 20–30% reduction in uterine weight in pregnant mice. On the other hand, NPs are relatively nontoxic to the kidney. Thus it becomes even more important to assess the toxicity of the NPs in different physiological systems. To assess the toxicity of the NPs, in vivo models are best choice as they mimic the biological system and thus their results are more reliable. Furthermore, proper techniques are also required to assess the toxicity of the NPs for particular organ system. Complete study of the toxicity potential of the NPs should be assessed as this will support the safe application of NPs.

Keywords Nanoparticles · Nanotoxicity · Systemic toxicity · Biokinetics · Toxicity assessment

7.1 Introduction

Nanoparticles (NPs) are defined as the particles with at least one dimension in the nanometer scale between 1 and 100 nm. During the last two decades, advances in nanotechnology have found wide applications of NPs in different fields like industrial, energy shortage, sensing, electronics, biomedical, bioengineering, and optical fields and even in the daily use commodities (Zhang et al. 2014). The increased applications of the NPs are because of their small particle size, novel physicochemical properties, and easy surface modification (Cho et al. 2009). Despite the large number of advantages that nanotechnology offers, the potential risk of NP exposure to human health is increasing (Pietroiusti et al. 2018). During the last decade, much attention has been paid to toxicity of NPs due to the same properties that make them useful. Early studies on asbestos and anthropogenic nanoparticles, such as from diesel exhausts, have shown that after daily exposure they can accumulate in the human body and can pose health risks, especially in occupational systems. Varoius health hazards from asbestos dust (like respiratory diseases, lung cancer, mesothelioma) have been recognized in workers involved in shipbuilding trades, asbestos mining and milling, manufacturing of asbestos products, insulation work in the construction and building trades, brake repair, and a variety of other trades. Exposure of diesel exhaust to susceptible animal models is also known to cause exacerbation of atherosclerosis (Bai et al. 2011) and changes in cardiac function (Carll

et al. 2012). Due to their nanometer dimensions, both natural and synthetic NPs act like biological molecules and can affect different mechanisms operating in the cell. NPs bind to different biomolecules in cells, such as DNA, lipids, and proteins (Zhang et al. 2014) and may affect their function. NPs may interact with the genetic material (DNA) and produce genotoxicity (Magdolenova et al. 2014; Li et al. 2014; Kwon et al. 2014). NPs may also interact with proteins involved in DNA replication, transcription, or repair (e.g., C60 fullerene binds to human DNA topoisomerase IIα in the ATP binding domain, which might inhibit the enzyme activity) (Baweja et al. 2011). Moreover, NPs can also cause indirect damage to DNA by toxic ions released from soluble NPs or reactive oxygen species generation (Magdolenova et al. 2014; Annangi et al. 2016; Manke et al. 2013). Long-term and short-term toxicity caused by the NPs to humans and animals has already become a serious concern. Recent studies have focused on the impact of NPs on biomolecules, cells, organs, or isolated physiological systems. These studies provide understanding of the mechanisms and level of the toxic impacts posed by the NPs. However, no organ or system is an isolated target for the NPs as the different organs of the body have similar basic properties. Thus the NPs can easily translocate between different organs, and signals can be transmitted across physiological boundaries. Therefore, it becomes extremely important to understand the nanotoxicity at the whole body level. For example, inhalation of multiwalled carbon nanotubes (MWCNTs) resulted in the release of signals from the lung that activated cyclooxygenase enzymes in the spleen and mediated immune suppression in mice (Mitchell et al. 2009). In another study, the exposure of cobalt chromium NPs to pregnant mice caused DNA damage in neonatal blood without accumulation of cobalt chromium NPs in the body of neonates (Sood et al. 2011). These results clearly indicate that NP toxicity cannot be limited to particular organ and NPs may have effects on the remote organs or tissues.

Thus the assessment of nanotoxicity becomes extremely important, and it requires a set of rules. Fischer and Chan (2007) emphasized the importance of developing predictive models for the assessment of nanotoxicity, while other researchers focused on histological changes (Kim et al. 2003) and pharmacokinetic parameters like exposing (Leite-Silva et al. 2013), biodistribution (Balogh et al. 2007; Goel et al. 2009), biochemistry metabolism, and clearance. However, exploration of nanotoxicity remains superficial in sacrificed animals. Based on the in vivo results of NP pharmacokinetics, including homeostasis regulation, systemically evaluating the impact of NPs on the major systems, including the hepatic, renal, digestive, pulmonary, hematological, cardiovascular, nervous, and immune systems, may provide profound insight into this field.

In the present book chapter, concise description about different in vivo animal model systems, the biokinetics (systemic assessment, absorption, distribution, metabolism, and excretion) of NPs, current knowledge about the toxicity of the NPs to different physiological systems, and different methods to assess the toxicity of the NPs for different organ systems using examples of in vivo systems are presented. This chapter will help in understanding the potential toxicity risks related to the NPs. This chapter provides important information about the potential health

hazards of NPs and thus will help in supporting the application of NPs by minimizing the adverse effects of NPs in vulnerable population.

7.2 Animal Models for Nanotoxicity Assessment

To study the effect of toxicity of NPs on different physiological systems, researchers have developed numerous in vitro toxicity models. However, the problem with in vitro assays is that the results obtained through these assays are inconsistent or conflicting with the in vivo studies due to the interference of NPs with either the detection system or the assay materials, and thus, the researchers are focusing on the development of more in vivo models for the toxicology studies (Bahadar et al. 2016).

For the in vivo studies of nanotoxicity, selection of suitable animal model is an important step. Although many animal models have been developed to study the nanotoxicity of different NPs (Table 7.1), a standard and predictive animal model is still unknown. The most common experimental animals used for the in vivo studies of nanotoxicity are murines (Lopez-Chaves et al. 2018; Bai et al. 2010). Besides mice and rats, zebrafish (Vandhana et al. 2015; Fang et al. 2015), rabbits (Leonardi et al. 2015), *Caenorhabditis elegans* (Zanni et al. 2012), and pig (Kim et al. 2004; Wu et al. 2009) have also been used in nanotoxicology studies. Mice and rats would be more appropriate models in nanotoxicity investigations as their genome is explicitly known and they have developed application in toxicity investigation. Assesment of the in vivo nanotoxicity can be divided into two main categories: the first involves tissue structure changes (Guo et al. 2013; Sayes et al. 2007), apoptosis (Sarhan and Hussein 2014; Coccini et al. 2013), and inflammatory changes in main organs (kidney, spleen, lung, brain, and heart), while the second one targets certain systems, whose structural specificities are likely to concentrate NPs. For example, hepatic sinusoid and Kupffer cells are the basic structures for liver function in metabolism and detoxication, where NPs are liable to be deposited. Consequently, appropriate nanotoxicity assessment models could be established by combining the exposing ways (ingestion, injection, transdermal delivery, and inhalation) and applications, especially for the drug delivery of NPs (Gross et al. 2015). On physiological basis, nanotoxicity can be tested from pathological changes, including morphological changes (gross, microscopic, and ultrastructural changes) and functional damage.

7.3 Biokinetics of Nanoparticles

Physicochemical properties of NPs like size (De Jong et al. 2008; Ma et al. 2011), shape (Qiu et al. 2010), aspect ratio (Qiu et al. 2010), surface charge, constituent (Hsieh et al. 2014; Mosqueira et al. 2001), surface chemistry, and aggregation status also affect absorption, distribution, metabolism, and excretion of NPs. These physicochemical properties determine the toxicity of NPs to different organ systems

Table 7.1 Distribution of different types of nanoparticles in animal models

Nanoparticle	Animal model	Distribution of nanoparticle	References
Gold	Mice	Liver, spleen, intestine, heart, skin, lung, dorsal root ganglion	Bahamonde et al. (2018), Sykes et al. (2014), and Koyama et al. (2015)
	Rat	Liver, intestine, spleen, kidney, feces, urine	Bahamonde et al. (2018), Lopez-Chaves et al. (2018), and Loeschner et al. (2014)
	Zebrafish	Embryo	Kim et al. (2013)
Titanium	Mouse	Spleen, lung, kidney, brain, heart, embryo	Wang et al. (2008), Wu et al. (2009), and Jia et al. (2017)
	Pig	Skin	Wu et al. (2009)
	Zebrafish	Reproductive system, embryo, nervous system	Sheng et al. (2014)
Manganese	Rat	Central nervous system, muscle, liver, kidney, bone marrow	Elder et al. (2006), Singh et al. (2013), and Nosrati et al. (2014)
Silver	Rat	Liver, kidney, spleen, testis, lung	van der Zande et al. (2012) and Johnston et al. (2010)
	Zebrafish	Liver, embryo, nervous system	Bar-Ilan et al. (2009), Choi et al. (2010a), and Xin et al. (2015)
Iron	Mice	Brain	Wang et al. (2009)
	Rat	Kidney, liver, spleen, lung	Kumari et al. (2012) and Zhu et al. (2008, 2009)
	Caenorhabditis elegans	Reproductive system	Qu et al. (2011) and Gonzalez-Moragas et al. (2017)
SWCNTs	Mice	Liver, spleen, heart, lung, kidney, stomach	Liu et al. (2008)
MWCNTs	Mice	Testis	Bai et al. (2010)
CNTs	Zebrafish	Brain, gonads, embryo	Li et al. (2015a, b) and Cheng and Cheng (2012)

SWCNTS single-walled carbon nanotubes, *MWCNTs* multiwalled carbon nanotubes, *CNTs* carbon nanotubes

(Table 7.2). Since the cell membrane is negatively charged, cationic NPs readily bind to the cell surface and are internalized, e.g., polyethyleneimine (cationic) coating of silica NPs increases the cell uptake as compared to neutral or anionic NPs (Xia et al. 2009). But cationic NPs exhibit higher toxicity than neutral and anionic nanoparticles, as the cationic NPs may damage the cell membrane and, after internalization, damage the lysosomal compartment (Xia et al. 2009). Modification of the NPs with ligand molecules that recognize specific cell membrane receptors also enhances their uptake into cells (Nel et al. 2009) and stability of the NPs.

Table 7.2 Effect of the main physicochemical properties of nanoparticles, as well as the routes of administration on the biodistribution of nanoparticles in animal models

Nanoparticle	Surface chemistry	Size/nm	Animal model	Administration route	Major observations	References
Polystyrene microspheres	Without modification	50, 100, and 300	Rat	Gavage	Accumulation in the liver and spleen via lymph	Jani et al. (1990)
MnO_2	Without modification	30	Rat	Whole body inhalation	Accumulation in CNS via olfactory bulb	Elder et al. (2006)
MWCNTs	Carboxylated and aminated	20–30 × 0.5–2 μm	Mouse	Intravenous	Accumulation in the testis	Bai et al. (2010)
SWCNTs	Without or coated by paclitaxel (PTX)–polyethylene glycol (PEG)	1–3 × 100 (diameter × length)	Mouse	Intravenous	Accumulation in the liver and spleen, less in the heart, lung, kidney, stomach, intestine, muscle	Liu et al. (2008)
TiO_2	Without modification	10, 25, and 60	Mouse	Intranasal instillation	Accumulation in the brain through olfactory bulb	Wang et al. (2008)
TiO_2	Hydrophobic or hydrophilic	80, 155	Hairless mouse	Dorsal skin exposure	Accumulation in the spleen, lung, kidney, and brain	Wu et al. (2009)
CdTe (CdSe) core (shell) type II QDs	Oligomeric, phosphine	10 (naked); 18.8 (coated)	Pig	Intradermal	Accumulation in sentinel lymph node	Kim et al. (2004)
Gold	Without modification	2, 40	Mouse	Intraperitoneal and intravenous	Macrophage uptake in the liver, less in spleen, small intestine, lymph nodes	Sadauskas et al. (2007)
Gold	Without modification	10–250	Rat	Intravenous	NPs of 10 nm entered the testis and brain	De Jong et al. (2008)

SWCNTS single-walled carbon nanotubes, *MWCNTs* multiwalled carbon nanotubes, *QDs* quantum dots

7.3.1 Absorption

Human exposure to the nanoparticles is increasing with the increasing applications of the NPs in different fields. NPs may be exposed to the body of human through different routes like skin, oral, intravenous, and respiratory (Fig. 7.1). Exposure to the NPs may be accidental or intentional. Accidental exposure to the nanoparticle includes exposure through respiratory, oral, or dermal as in case of pollution. Intentional exposure to the nanoparticle is by cosmetics, medicinal products mainly like implantation (Wennerberg et al. 2011), intravenous injection, oral

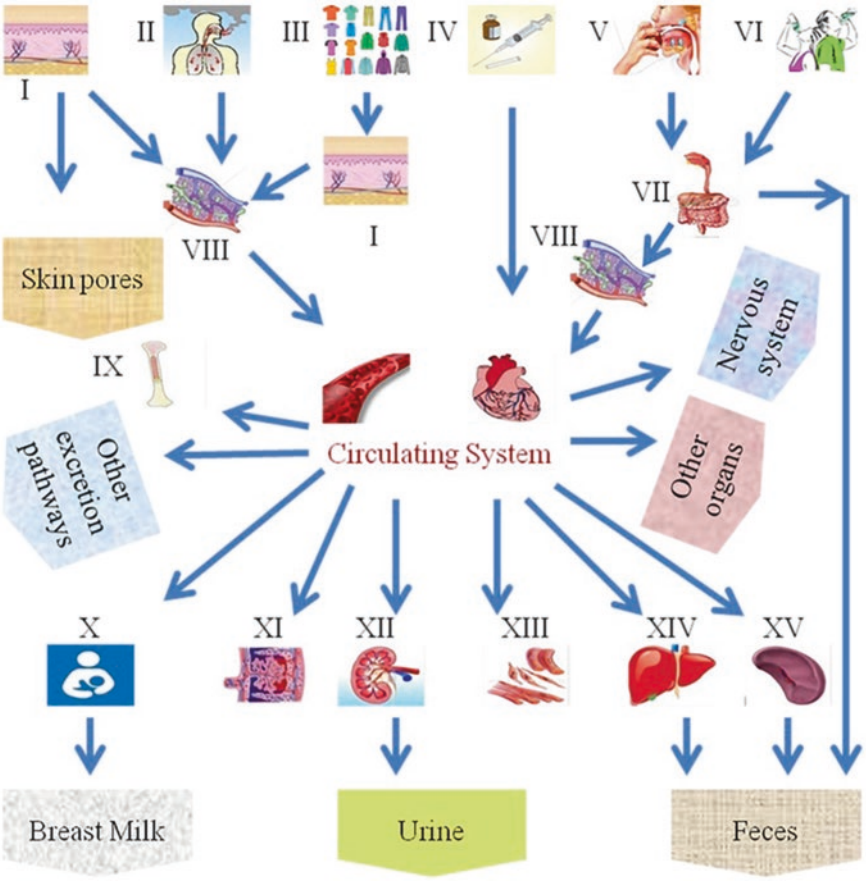

Fig. 7.1 Schematic of exposure of nanoparticles through different routes in the human body and distribution and excretion of the nanoparticles from the body. (I) Skin, (II) inhalation, (III) fabric, (VI) intravenous injection, (V) food intake, (VI) water intake, (VII) gastrointestinal tract, (VIII) lymph, (IX) bone marrow, (X) breast milk, (XI) placenta, (XII) kidney, (XIII) muscles, (XIV) liver, and (XV) spleen. (Reproduced from Brohi et al. 2017, Front Pharmacol 8:606. doi:https://doi.org/10.3389/fphar.2017.00606 under Creative Commons Attribution License (CC BY))

administration, inhalation, and transdermal use of pharmaceuticals. The skin prevents the absorption of larger NPs, but smaller NPs can cross the dermal barrier and translocate to different organs (Wu et al. 2009). Functionalization of the NPs' surface by the ligands that can bind to the cell surface receptors may enhance their absorption and internalization in the cells (Nel et al. 2009). Once the NPs are absorbed through different routes, they may enter the bloodstream and are distributed to different organs (Fig. 7.1). NPs easily enter the organs that have rich supply of blood, such as the liver and spleen, and are effectively retained by the reticuloendothelial system (RES) of these organs (Sadauskas et al. 2007; Liu et al. 2008). In different organs of the body, these nanoparticles are partially metabolized, excreted, or retained (Fig. 7.1).

7.3.2 Biodistribution

Exposure of experimental animal's skin to NPs results in the absorption of only a small number of NPs in the blood. However, smaller NPs (less than 100 nm) can penetrate the skin through different routes like intercellular, intracellular, or follicular and then translocate to various organs after being absorbed in the blood (Wu et al. 2009). NPs are absorbed in the blood after crossing different barriers, by oral administration, inhalation, injection (intravenous, intramuscular, subcutaneous, intradermal), and implants, and are distributed to different organs (Fig. 7.1). The translocation of NPs to different organs is a complicated process. For example, after dermal or gastrointestinal exposure, ultrafine particles (less than 10 nm) can enter the circulation via lymph node-mediated process (Jani et al. 1990; Kim et al. 2004). After inhalation, NPs can enter different systems by taking four possible paths: to lymph nodes, to the gastrointestinal tract, to the central and/or peripheral nervous system, and to the blood circulation (Oberdorster et al. 2005). The ingested quantum dots (QDs) may partly be translocated to the reproductive system in *Caenorhabditis elegans* (Qu et al. 2011). Translocation of NPs to different organs of the body is also, partially, controlled by physicochemical properties such as surface charge, size, shape and chemical properties (Choi et al. 2010b).

Understanding the biodistribution of NPs in different organs is useful to guide the adjustment and modification of NPs. The most common way of investigating the distribution of the NPs is to collect the organs (Balogh et al. 2007) or tissues such as the skin, lung, liver, kidney, heart, spleen, brain, and bone marrow of the animals after the animal sacrifice. NPs can be tracked by using detection methods based on their characteristics. For some metal NPs, their intrinsic properties could be probed by specific instruments. For example, gold composite nanodevices in mouse tumor tissues were explored by instrumental neutron activation analysis (Balogh et al. 2007). Silicon and cadmium (Guo et al. 2013) concentrations could be determined by inductively coupled plasma optical emission spectrometry. For some NPs, radiolabeled and fluorescent-labeled skills have also been used. [^{3}H]-PLA (Mosqueira et al. 2001) was used as a marker to show blood concentration and organ

distributions of poly(ethylene glycol)-grafted nanocapsules quantitatively in mice. Some fluorescent-labeled particles (Semete et al. 2010) in tissue homogenates were analyzed by plate reader. In materials like quantum dots with heavy metal cores (Li et al. 2015), it was more appropriate to apply multiplexing and multicolor imaging through single-particle Förster resonance energy transfer assays (Massey et al. 2015). Other imaging techniques, like magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography, optical imaging, and ultrasound, were introduced in the latest reviews (Janib et al. 2010; Park et al. 2015; Yang et al. 2016). Some of the imaging contrast agents have potential unintentional toxicity; these tracing methods may aggravate the toxicity of NPs. Moreover, the combination of contrast agents may alter the characteristics and consequently change the biodistribution of NPs.

7.3.3 Metabolism

Recently a number of articles have described in vivo metabolism of NPs (Wang et al. 2013a; Feliu et al. 2016). The physiological conditions in the cell compartment (acidic environment in endosomes) or organ (the acidic environment in the stomach, Meng et al. 2007) and the degradation by enzymatic catalysis in cell lysosomes may play primary roles in the metabolism of NPs. The major route for the entrance of nanoparticle into the cells is endocytosis. During endocytosis, the NPs are enclosed by early or late endosomes having slightly acidic pH (6.2–6.5) or pronouncedly acidic pH (4.5–5.5), respectively. Some metallic nanoparticles, such as silver (Arora et al. 2008), quantum dots (Hoshino et al. 2004; Gao et al. 2004), and iron oxide NPs may undergo dissolution in the acidic microenvironment in endosomes and release metallic ions (Zhu et al. 2011). For majority of the biological applications, the NPs are injected intravenously, and in the biological environment, the NPs are exposed to large number of biomolecules such as lipid, protein, sugars, ions, and metabolites in the body (Feliu et al. 2016). These biomolecules adsorb on the surface of NPs and form biomolecule corona or protein corona (Wan et al. 2015). These biomolecules are easily degraded by the enzymes in endosomes and lysosomes and macrophages of the lung, liver, and spleen (Wang et al. 2013a) and release the inorganic part. NPs made up of inorganic ions like Ag, ZnO, CdSe, and FeOx corrode and release metal ions (Soenen et al. 2015), whereas gold NPs are inert and thus stable against degradation. Degradation of such NPs occurs by the strong binding of the ligands such as thiols (available from glutathione) to the surface of gold NPs. Under certain conditions, this may lead to pulling out of atoms via the ligand from the surface of gold NPs (Paulsson et al. 2009) leading to slow degradation of gold NPs. In some cases, the released ions might be more toxic in some cases. This complicates the evaluation of nanoparticle toxicity as in the case of some quantum dots, most of which are made of toxic heavy metals (Derfus et al. 2004).

7.3.4 Excretion

Nanoparticles can be partially excreted through urine, feces, or breast milk (Fig. 7.1). The physicochemical properties of the NPs also affect their excretion pathways (Zhang et al. 2014), e.g., single-walled carbon nanotubes (SWCNTs) coated with polyethylene glycol are mainly cleared through feces and urine (Liu et al. 2008), while liposomes are eliminated via the hepatobiliary pathway (Alexis et al. 2008). NPs with diameter less than 10 nm can be eliminated in urine (Longmire et al. 2008; Naz et al. 2016), whereas NPs having diameter larger than 80 nm are trapped by the liver and spleen and are slowly excreted in feces (Alexis et al. 2008). NPs that are degradable in macrophages may be cleared by the reticuloendothelial system (RES) organs, while nondegradable particles may be deposited in organs for a long time (Zhao et al. 2011a). The possibility of other excretion pathways, like saliva, sweat, and breast milk, cannot be ruled out (Li et al. 2010). Thus it is very much clear that the NPs are only partially excreted, and some organs retain the NPs for long periods. This persistence of the NPs for extended period prolongs the impact on physiological systems and thus poses a considerable risk to health, i.e., nanotoxicity.

7.4 Nanotoxicity and Its Assessment in Different Physiological Systems

7.4.1 Pulmonary Nanotoxicity and Its Assessment

The human respiratory tract consists of three sequential regions: nasopharyngeal, tracheobronchial, and the pulmonary regions. Alveoli and airways of the respiratory tract have vast internal surface area (approximately 150 m^2) which facilitates broad access of inhaled materials to the lung tissue (Bakand et al. 2012). Inhaled NPs are reported to cross alveolar epithelial and vascular endothelial cell layers (Heckel et al. 2004). These NPs are transported from the lungs to different organs through the lymphatic vessels and blood (Kreyling et al. 2010) and may result in toxicity to the whole body. Respiratory nanotoxicity can be divided into two main categories: respiratory toxicity to the lungs and systemic toxicity to the organs/systems other than the respiratory tract.

Nanotoxicity to the Lungs The lung is one of the primary sites for the accumulations of the NPs because it is a reticuloendothelial system. The respiratory toxicity of NPs has been investigated mainly after exposure of animals to NPs by inhalation. Inhalation of different NPs leads to different types of lesions in the lungs depending upon the properties of the NPs. Inhalation of MWCNTs (0.5 and 2.5 mg m^{-3}) by rats resulted in pronounced multifocal granulomatous inflammation of the lungs (Ma-Hock et al. 2009). Inhalation of carbon nanofibers causes the formation of extrapulmonary fibers and inflammation in the terminal bronchioles and alveolar

ducts of rats (DeLorme et al. 2012). Exposure of NPs by dermal route resulted in the slight increase in the alveolar thickness in the hairless mice (Wu et al. 2009). Inhalation of TiO_2 NPs causes lung inflammation in mice (Lindberg et al. 2012). Inhalation of gold NPs by rats showed that the gold NPs can be translocated from the lung to organs such as the kidney, aorta, spleen, and heart and causes damage to the lung and these organs (Yu et al. 2007; Takenaka et al. 2006).

Apart from inhalation exposure, intratracheal instillation is another method for the exposure of the NPs to the pulmonary systems. This method delivers a higher dose to animals, in comparison to the inhalation (Morimoto et al. 2012). NPs administered by the intratracheal instillation are taken up by alveolar macrophages and alveolar epithelial cells. NPs that enter the alveolar epithelium are cleared from the lungs with difficulty (Zhu et al. 2009). Administration of various NPs like carbon nanotubes (CNTs) (Chou et al. 2008), carbon black (Bourdon et al. 2012), silver (Park et al. 2011), and iron oxide (Ban et al. 2012) through the intratracheal instillation route induced pulmonary inflammatory responses, granuloma formation (Muller et al. 2005; Chou et al. 2008; Shvedova et al. 2005), and fibrotic lung injury (Muller et al. 2005). In Sprague Dawley rats, TiO_2 nanorods generated hydroxyl radicals in the lungs and caused reversible pneumotoxicity with no significant alterations in pulmonary immune function (Roberts et al. 2011). Single intratracheal instillation of iron oxide NPs resulted in the suppression of local immunity in the mice (Ban et al. 2012).

Systemic Nanotoxicity In addition to causing local injury to the lungs, NPs can be transported to other organs by the lymphatic system and circulatory system, leading to systemic toxicity. The translocation of NPs to other organs from the lungs depends on the physicochemical properties of the NPs. The translocation capability of organic and inorganic NPs is affected by the size, surface charges (Choi et al. 2010b), and surface area (Schmid and Stoeger 2016) of the NPs. After instillation exposure, NPs having size less than 6 nm are freely absorbed in the circulation in rats, NPs having size smaller than 34 nm are translocated to the mediastinal lymph nodes, while cationic NPs tend to be retained in the lungs. Long fiber-like NPs like MWCNTs (more than 20 μm in length) were translocated to the pleural space from subpleural alveoli (Murphy et al. 2011).

Inhalation of NPs may also damage the innate immunity of an animal. After inhalation of MWCNTs, an impaired immune response to sheep erythrocytes and decreased activity of natural killer cells were found in mice (Mitchell et al. 2007). MWCNTs also stimulate the release of immunoregulatory factor TGF-β from the lung and cause humoral immune suppression (Mitchell et al. 2009). Fe_2O_3 and ZnO NPs accumulated in the liver and lungs after inhalation and resulted in changes in biochemical markers of blood in rats (Wang et al. 2010). Susceptibility of the individual to nanotoxicity of the airborne particles also depends on age (Chen et al. 2008), genetic susceptibility, and cardiovascular disease status (Ge et al. 2012). After 24 hours of intratracheal instillation, SWCNTs induced local inflammatory responses and oxidative stress in lungs, perivascular myocyte degeneration, and

peripheral vascular lesions, in hypertensive rat model (Ge et al. 2012). Inhalation of TiO_2 NPs (21 nm) compromised the dilation of the carotid artery in rats. This response is similar to pathophysiological microvascular changes occurring in chronic diseases such as diabetes, hypertension, and heart failure (Nurkiewicz et al. 2008). Inhaled Fe_2O_3 and ZnO NPs accumulated in the liver and lungs and caused changes in blood biochemical markers in rats (Wang et al. 2010). Gold nanoparticles had also been shown to cause systemic damage to distal organs (Takenaka et al. 2006).

In summary, NPs exposed to the respiratory system are retained by the lung reticuloendothelial system and the alveolar epithelium. Respiratory exposure also results in the absorption of NPs to the blood circulation in the lung alveoli. NPs less than 6 nm are readily distributed to the blood, while NPs more than 34 nm are distributed via lymphatic systems. NPs cause pulmonary inflammatory responses, fibrosis, and granuloma formation in the lungs. Systemic toxicity of NPs absorbed by the respiratory system affects immune responses and systemic microvascular function in animal models and the toxicity depends on the characteristics of the NPs. Inhaled TiO_2, ZnO, and Fe_2O_3 NPs are more toxic to lungs and liver due to their accumulation in these organs.

Nanotoxicity Assessment For in vivo assessment of the toxicity of NPs in the pulmonary system, accumulation of the NPs in different tissues of the pulmonary tract and changes in biochemical parameters, histology, and genotoxicity are studied by different methods (Armstead and Li 2016). The most common method to study pulmonary nanotoxicity is intratracheal instillation for long-term (Bermudez et al. 2002, 2004) and short-term (Ma-Hock et al. 2009) toxicity assessments, followed by the bronchoalveolar lavage (Paranjpe and Müller-Goymann 2014; Armstead and Li 2016) to collect the fluid for in vitro biochemical analysis, including lactate dehydrogenase assay. Lactate dehydrogenase index is useful to indicate pneumocyte injury (Yang et al. 2016). To study the inflammatory reactions occurring in the respiratory tract, the number of bronchoalveolar lavage fluid-recovered neutrophils (Zhang et al. 2002) is also counted to indicate the extent of the inflammation. Hematoxylin and eosin (H&E) staining of the lung tissue of the hairless mice exposed to TiO_2 NPs showed slight alveolar thickness on obervation at 100 X under the microscope (Wu et al. 2009) (Fig. 7.2). In order to study the oxidative stress of pulmonary system, lipid peroxidation (LPO) and glutathione production assays are conducted in the bronchoalveolar fluid (Braakhuis et al. 2014). Bronchoalveolar lavage fluid can also be used to evaluate the pulmonary toxicity of NPs by studying other biochemical parameters like total protein and albumin, IL-1, TNF-α, fibronectin, or cystatin-C and nitric oxide synthase (Armstead et al. 2015; Armstead and Li 2016). To detect pulmonary accumulation of NPs, MRI was used to visualize antibody-conjugated superparamagnetic iron oxide NPs in a lipopolysaccharide-induced chronic obstructive pulmonary disease mice model (Al Faraj et al. 2014).

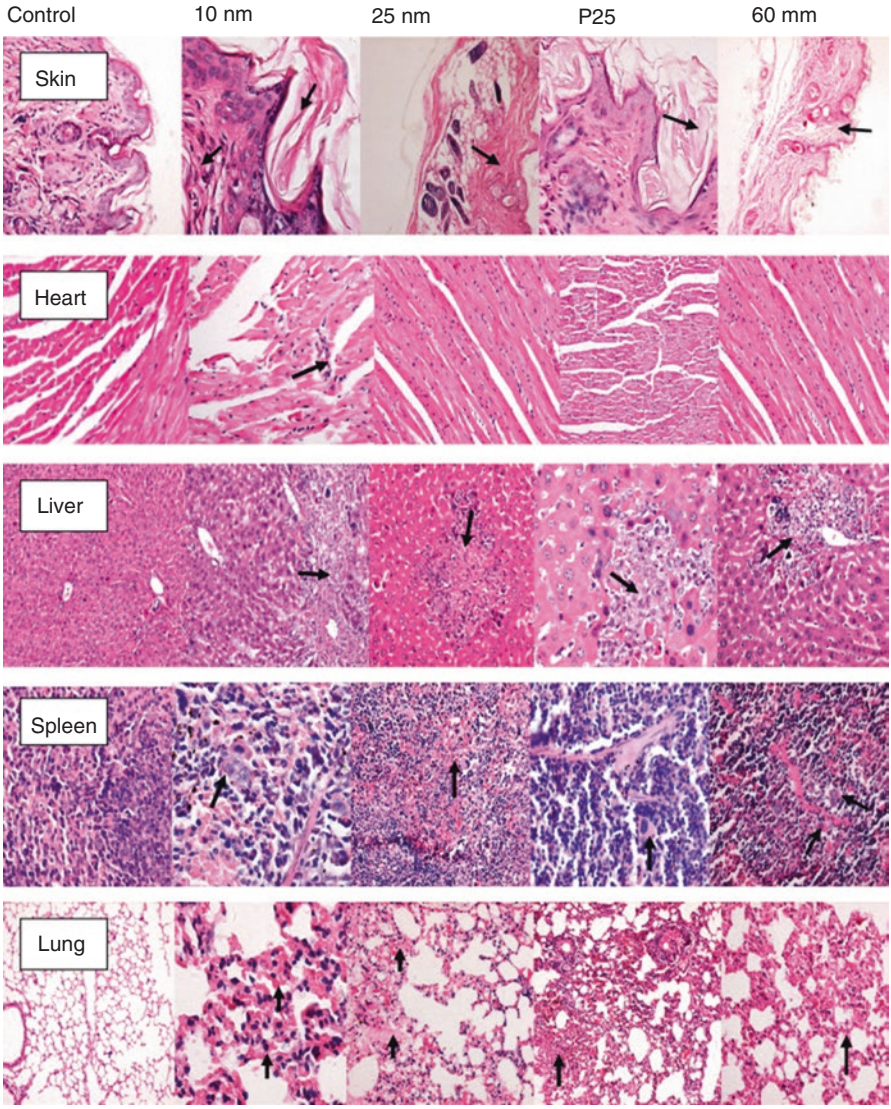

Fig. 7.2 Histopathological evaluation of the organ of hairless mice after dermal exposure to TiO_2 nanoparticles of different sizes for 60 days. Samples were stained with hematoxylin and eosin (H&E) and observed at 100×. The arrows point at pathological changes in various tissue sections. Skin sections from all 10 nm and Degussa P25 (21 nm) nanoparticle treatment groups showed excessive keratinization, thinner dermis, and an epidermis with wrinkles. In the liver, focal necrosis (25 nm, Degussa P25, 60 nm) and liquefaction necrosis (10 nm) are observed. In heart tissue sections, only small traces of white blood cells are observed in the 10 nm group. In the spleen and lung tissues, increased proliferation of local macrophages and slight alveolar thickness are observed, respectively. (Reproduced with permission from Wu et al. 2009, Toxicol Lett 191:1–8. doi:https://doi.org/10.1016/j.toxlet.2009.05.020)

7.4.2 Cardiovascular Nanotoxicity and Its Assessment

Nanoparticles absorbed in the blood are transported to distal organs (Jun et al. 2011; Corbalan et al. 2012). Cardiac uptake of NPs was revealed by de Barros et al. (2014) using a radioisotope [^{125}I] of iron oxide NPs. During the transportation of the NPs, the fluid dynamics of blood is altered (Buxton 2008); NPs affect the walls of the blood vessels (Decuzzi et al. 2005) and adhere to the walls of the blood vessels by nonspecific interactions like van der Waals forces and electrostatic and steric interactions (Zhang et al. 2014). This is related to the physical properties of NPs like size and shape (Liu et al. 2012; Shah et al. 2011), e.g., oblate-shaped NPs showed a higher adhesion probability to the walls of the blood vessels than spherical NPs of the same volume (Decuzzi and Ferrari 2006). In blood, the circulating NPs get coated by the different biomolecules like lipids, proteins, and sugars and form biomolecule corona (Demir et al. 2011; Wan et al. 2015). Protein corona is the most common biomolecule corona (Wan et al. 2015) and influences in vivo absorption, distribution, and metabolism of the NPs (Monopoli et al. 2012). Protein adsorption increases the stability of the NPs and also improves the biodistribution of the NPs and also increases the cellular accumulation of the NPs (Demir et al. 2011). Toxicity of the NPs to the circulatory system occurs in different ways. Intravenous injection of NPs will have certain impact on the cardiovascular system. After inhalation, different NPs in the lungs of animal models stimulated oxidative stress and release of proinflammatory mediators and coagulation factors in the blood leading to the cardiovascular lesions (Donaldson et al. 2001). In apoprotein E-deficient (ApoE $^{-/-}$) mouse model, NPs like carbon nanotubes, carbon black, and nickel hydroxide increased plaque formation in ascending aorta and thoracic and abdominal aorta and exacerbated the condition of atherosclerosis (Kang et al. 2011; Vesterdal et al. 2010; Sun et al. 2005). Exposure of carbon black NPs by the intratracheal route for 10 weeks, exacerbated atherosclerotic lesions in low-density lipoprotein receptor knockout (LDLR/KO) mice (Niwa et al. 2007). NPs in the circulation activate coagulation pathways and thrombosis (Zhang et al. 2014). For example, MWCNTs with different surface chemistries (pristine, carboxylated, and amidated) caused platelet activation and blood coagulation in mice (Burke et al. 2011). Silver NPs induced hemolysis, membrane injury, and lipid peroxidation in a size- and dose-dependent manner in fish (Chen et al. 2015). Carbon nanoparticles (Radomski et al. 2005) and injected silver nanoparticles (Jun et al. 2011) cause increased platelet aggregation and thus vascular thrombosis in a dose-dependent manner (Vermylen et al. 2005; Hoet et al. 2004). Silver NP exposure in rats elevated the level of serum total cholesterol, triacylglyceride, and low density lipoprotein cholesterol and produced increased inflammation and cellular degeneration in the heart (Sulaiman et al. 2015).

In summary, NPs absorbed through the different routes reach the circulatory system. NPs alter the fluid dynamics of blood, increase intracellular oxidative stress, and induce inflammation that leads to cardiovascular lesions, accumulation of cholesterol in the aorta and heart, penetration of WBCs in the heart and blood vessels, thrombosis, platelet aggregation, and cardiovascular malfunction in experimental animals.

Toxicity Assessment For the assessment of NP toxicity, hematology and serum drew lots of attention. Phlebitis is the first and most common clinical observation. Typical symptoms of phlebitis can be seen through pathological sections (Laverman et al. 2001). Hemolysis and thrombosis are other common risks of the NP toxicity. Hemolysis is usually tested in vitro (Fornaguera et al. 2015), while the vascular thrombosis is studied in animal models like rats (Radomski et al. 2005). Generally, the toxicity of the NPs in circulation is evaluated by studying the variation in complete blood parameters, including erythrocytes, total leukocytes, hemoglobin, and hematocrit (Sarhan and Hussein 2014). Cardiac injury by the NPs is assessed by the analysis of serum markers, like troponin-T, creatine kinase-MB, and myoglobin (Baky et al. 2013). To assess the damage to the contraction function of the cardiac system, cardiac calcium concentration and DNA damage can also be analyzed (Yang et al. 2016). Oxidative stress biomarkers including lipid peroxidase, reactive oxygen species, and antioxidant enzymes like superoxide dismutase, glutathione peroxidase, and catalase are also studied by different workers to know the damage to the cardiac cells (Shafiee et al. 2010; Sulaiman et al. 2015; Chen et al. 2015). Monitoring of the cardiovascular parameters like arterial pressure and heart rate telemetrically can also be used for the energetics study involving nanocarriers (Vlasova et al. 2014).

7.4.3 Hepatic Nanotoxicity and Its Assessment

The liver is a multifunctional organ, and it plays main role in clearing xenobiotic chemicals from the body and aids in digestion, synthesis, and storage of glucose, fatty acids, and iron and endocrine function. Thus, toxicity to the liver will have multiple consequences. Since the liver is the major reticuloendothelial system, it is the major organ for the accumulation of NPs (Hirn et al. 2010). NPs taken up by the hepatocytes and Kupffer cells (Fig. 7.3) are cleared through the hepatic biliary system. Long-term accumulation of NPs in the liver results in the toxicity of the NPs. After the intravenous and intraperitoneal injection of SWCNTs, NPs were partially excreted in feces (Derfus et al. 2004; Wang et al. 2004). TiO_2, carbon nanotubes, and SiO_2 NPs induce hepatotoxicity, as indicated by unusual serum levels of aspartate aminotransferase and alanine aminotransferase (Kim et al. 2010). Histopathological examination showed injuries to hepatocytes like necrosis, fibrosis, bile duct hyperplasia, congestive dilation of the central veins, and abnormal pigmentation (Hwang et al. 2012). Silver NPs caused necrosis and hemorrhage in the liver cells of rat (Wen et al. 2017). Poly(ethylene glycol)-coated gold NPs administered intravenously in mice accumulated in the liver and spleen cells of mice. The accumulated gold NPs caused inflammation and apoptosis in the liver (Cho et al. 2009).

Cytochrome P450 family of enzymes of the liver play major role in the metabolism of toxins and prevent the body from the injuries caused by these toxins. At low doses (IC_{50} less than 30 mg/ml), oral administration of silver NPs inhibited CYP2C

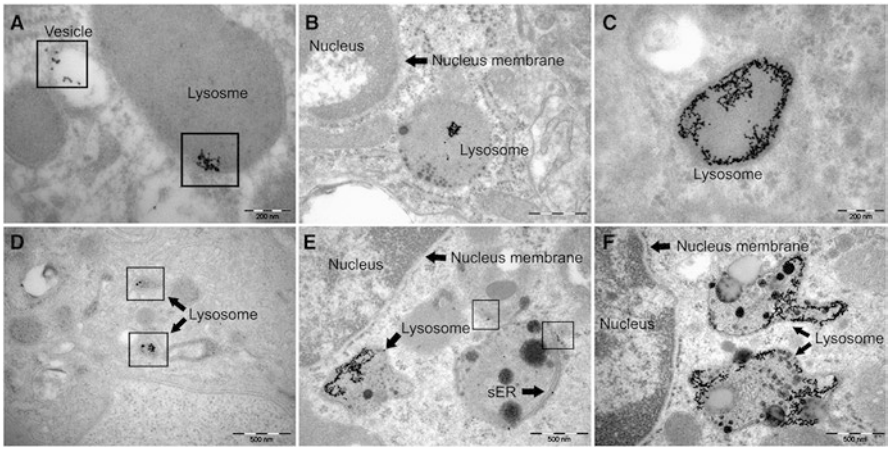

Fig. 7.3 Thin-section TEM images of mouse liver and spleen tissues at 24 h or 7 days after intravenous injection of PEG-coated gold NPs. The micrographs show entrapped PEG-coated gold NPs and their clustering and localization in intracellular organelles, such as lysosomes and smooth endoplasmic reticulum. Kupffer cells at (**a**) 24 h or (**b**, **c**) 7 days postinjection at scope magnifications of (**a**) 100,000×, (**b**) 50,000×, and (**c**) 100,000×. Spleen macrophages at (**d**) 24 h or (**e**, **f**) 7 days after injection at scope magnifications of (**d**) 50,000×, (**e**) 50,000×, and (**f**) 50,000×. (Reproduced with permission from Cho et al. 2009, Toxicol Appl Pharmacol 236:16–24, doi:https://doi.org/10.1016/j.taap.2008.12.023)

and CYP2D activity in rat liver microsomes (Kulthong et al. 2012). TiO_2 NPs caused oxidative stress to the hepatocytes and increased lipid peroxidation and decreased superoxide dismutase and glutathione peroxidase (GPx) level in the liver of rats (Orazizadeh et al. 2014). Fe_3O_4 NPs administration in the rat at higher doses (150 and 300 μg/g) caused elevation in liver enzymes (alanine transaminase, aspartate aminotransferase, and alkaline phosphatase) (Parivar et al. 2016). Intravenous injection of silver NPs also caused changes in the WBC count, platelet count, hemoglobin, and RBC count and the levels of liver function enzymes (Wen et al. 2017) in rats. These studies show that the toxicity of NPs becomes a matter of concern for individuals suffering from the liver diseases, obesity, and diabetes.

In summary, Kupffer cells and hepatocytes of the liver internalize NPs that reach to them through the circulatory system. NPs accumulate in the liver and cause hepatotoxicity and produce lesions like focal necrosis and liquefaction necrosis in the liver. NPs adversely affect the function of enzymes of cytochrome P450 family. The effects of NPs on the secretion of bile, synthesis of glucose and fatty acids, and protein metabolism are largely unknown. Thus, the persons suffering from liver diseases or with compromised liver are more vulnerable to the toxicity of the NPs.

Toxicity Assessment Hepatic nanotoxicity is usually assessed by the histopathological studies or changes in the blood parameters and liver function test. Immunohistochemistry and histopathological examination were used to detect liver fibrosis, fibrosis, bile duct hyperplasia, abnormal pigmentation, and inflammation

(Pan et al. 2012; Hwang et al. 2012). Hematoxylin and eosin (H&E) staining of tissue sections of the NPs also helps in understanding the focal necrosis and liquefaction necrosis in the hepatic tissue (Wu et al. 2009) (Fig. 7.2). Analysis of different enzymes related to the liver function evaluation, mainly including alanine transaminase, aspartate transaminase, alkaline phosphatase, superoxide dismutase (Yamagishi et al. 2013; Parivar et al. 2016; Wen et al. 2017), and c-glutamyl transferase (Cho et al. 2009), is also used to assess the hepatic toxicity of the NPs. If conditions allow, multiple automated hematology and chemistry analyzer are beneficial for the comprehensive analysis (Adamcakova-Dodd et al. 2014; Carneiro et al. 2013). Transmission electron microscopy of the tissue sections of the liver will also help in understanding the distribution and pathological lesions in the liver tissue. Distribution of poly(ethylene glycol)-coated gold NPs in the liver cells was demonstrated by using transmission electron microscopy (Fig. 7.3).

7.4.4 Renal Nanotoxicity and Its Assessment

The kidney is an important organ for filtration of the blood and eliminates the toxins from the body through urine. It also eliminates NPs through urine. Apart from the reticuloendothelial system, NPs also accumulate readily in the kidney. Renal glomerular basement membranes are fragile to toxic stimuli and thus prone to nanotoxicity. Larger NPs have higher tendency to accumulate in the kidney as smaller NPs are eliminated through urine (Jefferson et al. 2011). The glomerular basement membrane along with the podocyte foot processes apparently allows only NPs of approximately 10 nm size or molecular weight of 30–50 kDa (Zhang et al. 2014). However, there are few exceptions to this rule. SWCNTs can penetrate the physical barriers present in the kidney due to their needle-like shape and are excreted in urine. After intravenous injection of SWCNT NPs (0.8–1.2 nm in diameters and 100–500 nm in length) in mice, the NPs were detected in the bladders of mice within 1 min (Ruggiero et al. 2010). Some spherical nanoparticles much larger than the suggested "cutoff size" can also be excreted in the urine. Parenterally administered magnetic NPs (100 nm) were found in bladders of mice by magnetic resonance imaging, indicating that they are partially excreted in urine (Lacava et al. 2003).

Many studies have indicated that the kidney is relatively resistant or insensitive to the nanotoxicity. Intraperitoneal injection of naked gold NPs (12.5 nm) of different doses (40, 200, and 400 μg/kg/per day) to mice for 8 days caused no changes in urea nitrogen or creatinine level in blood (Lasagna-Reeves et al. 2010). However there are other studies which have shown the toxic effects of the NPs on the kidney. Kidney is one of the primary targets of copper NPs. Oral gavage of copper NPs (23.5 nm) at a dose of 232 mg/kg caused dose-dependent pathological changes, grave injury to the kidneys, and changes in the blood urea nitrogen level in a gender-dependent manner in mice. Male mice showed more severe symptoms than female mice (Chen et al. 2006). Renal glomerular swelling and histopathological lesions in the kidneys of mice were induced by TiO_2 at 1944 or 2592 mg/kg induced. But

blood urea nitrogen levels remained unchanged even at these high doses of TiO_2 NPs (Chen et al. 2009). Intraperitoneal injections of TiO_2 NPs did not change serum creatinine levels, but serum urea and uric acid showed significant changes, and deposition of hyaline-like materials, inflammation, dilatation of Bowman's capsule, and degenerations were observed on histopathological examination, though the effects were temporary and kidney function returned to normal (Fartkhooni et al. 2016). Intravenous injection of silver NPs in rats resulted in the red-colored urine, increased blood urea nitrogen level, and diffused hyaline degeneration in renal tubular epithelial cells (Wen et al. 2017).

In summary, the kidney is less sensitive to the NP toxicity as compared to other organs. The kidney prevents the excretion of NPs having size more than 410 nm in urine because of the glomerular filtration barrier. But needle-shaped NPs may bypass this barrier and are excreted in urine. However silver, titanium, and copper NPs may cause renal toxicity and lesions in the kidney.

Toxicity Assessment Renal toxicity of the NPs can be best studied by the histopathology and serum blood urea nitrogen level estimation. Renal glomerular degeneration can be studied best by histopathological study. The different pathologic changes, like hyaline degeneration in renal tubular epithelial cells, inflammation, dilatation of Bowman's capsule, glomerulosclerosis, and collagenous tubulointerstitial matrix, can be confirmed by immunohistochemistry and histopathology by selecting different markers like by detecting fibrotic and mesenchymal markers transforming growth factor-β1, interferon-6, type I collagen, fibronectin, and vimentin (Coccini et al. 2015; Fartkhooni et al. 2016; Wen et al. 2017). Some special dyes, including periodic acid–Schiff, periodic acid–silver methenamine, and Masson, can be observed under light microscope (Yanardag et al. 2002). The pathological diagnosis can be also determined by electron microscopy. From a functional aspect, kidney indices (Gui et al. 2013) are commonly measured. Urine and blood parameter changes like protein in urine, hematuria, urine albumin, creatine ratio, serum or blood urea nitrogen, serum creatine, uric acid, etc. were also studied in vivo by different workers (Gandhi et al. 2013; Petrica et al. 2015; Fartkhooni et al. 2016) to evaluate the injury to glomerular filtration membrane.

7.4.5 Nanotoxicity to the Gastrointestinal System and Its Assessment

Oral administration of drugs or others is favorable over other methods due to convenience and compliance for patients. However, drug bioavailability through oral administration is limited because of physiological barriers of the gastrointestinal tract (GIT). Nanotechnology has improved the bioavailability of the drugs (Sonaje et al. 2011; Pridgen et al. 2014) by preventing biologicals from inactivation by acidic and alkaline environment and enzymatic degradation in the GIT.

The hazardous impact from oral uptake of NPs cannot be ignored because NPs are widely used in the industry as food additives and coloring agents, in food packaging, and even in other agents like toothpaste and drugs (Augustin and Sanguansri, 2009). Environmental pollution by NPs makes it possible that NPs frequently enter the digestive system through drinking water, street foods, and nanopharmaceutical products (Zhu et al. 2009). After the oral exposure/uptake of NPs, the stomach and intestines are the main organs in which NPs accumulate. NPs that are not absorbed quickly by the GIT are quickly eliminated in the feces (Loeschner et al. 2011). NP absorption in the gastrointestinal tract has been studied in different animal models. Orally administered NPs have been detected in distal organs though their absorption rate by the GIT is slow. Silver and TiO_2 NPs administered orally in rats and mice, respectively, accumulated in the wall of ileum as well as in the lung, liver, spleen, and brain (Loeschner et al. 2011; Wang et al. 2007). NP retention in the GIT may affect its structure and function. In chicken, acute oral exposure of polystyrene NPs to disrupted the iron transport in intestinal epithelial cells, while chronic exposure increased the surface area of intestinal villi available for iron absorption to compensate for the decreased iron transport caused by NP exposure (Mahler et al. 2012). NPs induce similar effects in lower invertebrate and vertebrate animal models. NPs of silver, CuO, TiO_2, and nickel accumulated in the digestive glands after oral exposure in *Nereis diversicolor*, *Mytilus galloprovincialis*, *Porcellio scaber* (Isopoda, Crustacea), *Mytilus edulis*, and zebrafish and induced oxidative stress and damaged digestive gland cell membranes (Valant et al. 2011; Tedesco et al. 2010; Gomes et al. 2012; Garcı'a-Alonso et al. 2011; Croteau et al. 2011; Novak et al. 2012). In rats the orally administered silver NPs accumulated in the lamina propria of the small and large intestine and also in the upper villi of the ileum and protruding surface of the fold in the colon and disturbed the mucus composition (sialylated mucins increased) in the goblet cells in the intestines, similar to ulcerative colitis and small intestine carcinoma (Jeong et al. 2010). Oral administration of high dose (5 g/kg) of ZnO NPs stimulated acute responses like anorexia, vomiting, and diarrhea in mice, and after 2 weeks, slight inflammation in the stomach and intestine was also noticed (Wang et al. 2006). Oral exposure of CeO_2 NPs to the zebrafish resulted in decreased body weight, delayed vertebral calcification, and injury to epithelial lining of GIT (Lin et al. 2014). Further investigations are required to establish the significance of these findings to the pathophysiology of the gastrointestinal tract. Additionally, NPs may have impact on the gut microflora on oral administration in the animals.

In summary, NPs after the oral route exposure mainly interact with the stomach and intestines. NPs that are not absorbed by the GIT are quickly eliminated in feces. The absorbed NPs accumulate in the stomach and intestine of the GIT. NPs accumulated in the GIT damage intestinal structure and disturb its functions. Depending on physicochemical nature, NPs absorbed through the GIT may enter the circulation and reach distant organs. In the GIT, NP toxicity may result in increased gastric emptying and altered nutrient absorption. However, how NPs affect the immunological defense capability of the intestine and whether they affect the gut microbiome or not remain unknown.

Toxicity Assessment For the toxicity assessment of NPs in GIT system, the initial step should be the histological assay to know about the GIT microvilli and epithelial atrophy (Lin et al. 2014; Han et al. 2012) under electron microscope. The number of mast cells in the stomach should be counted (Wang et al. 2013b) as indicator of the GIT epithelial damage. Additionally, functional tests of GIT should also be done to assess the damage to GIT. Evaluation of metal content and electrolyte could reflect the absorption function of GIT indirectly (Yang et al. 2016). Lin et al. (2014) developed a novel method to quantitatively monitor the digestion of intramolecular-quenched protein under fluorescence spectroscopy in zebrafish to show digestive malfunction and developmental abnormalities. Both serum proinflammatory (IL-1, IL-6, IL-12) and anti-inflammatory (IL-10, TGF-β) cytokines increased in the mice in response to silver NPs on oral administration (Park et al. 2010a). These may serve as marker of the NP toxicity on oral administration.

7.4.6 Nanotoxicity to the Nervous System and Its Assessment

Two physiological barriers, the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB), protect the human central nervous system (CNS) (Sharma and Johanson 2007) from the toxic effects of xenobiotics. These barriers also make the delivery of CNS therapeutics difficult. Owing to their small size, NPs are able to cross these barriers and reach the CNS making them potential therapeutic carriers for the treatment of CNS diseases (Bharali et al. 2005). At the same time, it raises concerns about their possible toxic effects on the CNS. There are at least three modes by which nanoparticles enter the CNS. First, NPs like CdSe/CdS/ZnS quantum rods coated with various biomolecules (Xu et al. 2008), iron oxide NPs coated with chitosan copolymer (Veiseh et al. 2009), and magnetic NPs coated with silica (Kim et al. 2005; Simko and Mattsson 2010) penetrate the BBB without damaging it. Second, NPs like silver, aluminum, and copper penetrate the BBB by disrupting its integrity. BBB disruptive effect of silver and copper NPs was most prominent in animals treated with aluminum NPs (Sharma et al. 2010). NPs also enhance stress-induced BBB disruption, inflammation, and damage to the cells of CNS. Silver and copper NPs (50–60 nm) aggravated the BBB breakdown induced by hyperthermia and produced severe cognitive dysfunction and brain pathology in rat and mice (Sharma et al. 2009a, b). Brain microvessel endothelial cells form the major component of the BBB. Therefore, CNS inflammation and functional abnormalities caused by NPs may be attributed to NP-induced damage to microvessel endothelial cells. Third, NPs like Fe_2O_3 and TiO_2 can bypass the BBB by translocating to the brain along the olfactory nerve pathway (Wang et al. 2008, 2009).

NPs that affect the CNS cause the behavioral changes in the animals as first symptom. In rats, intraperitoneal injection of silver, copper, or aluminum NPs resulted in mild to moderate deficits in mental and sensory–motor functions, as demonstrated by poor performance in tests like rotarod, grid walking, inclined plane angle, and footprint analysis. More malfunctions were observed in animals on

administration of NPs by intravenous, intracarotid, or intracerebroventricular routes than intraperitoneal administration, indicating that NPs disrupt the BBB and induce brain damage (Sharma and Sharma 2007, 2010).

NPs may also cause changes in the level of dopamine, neurotransmitter involved in the regulation of movement and emotional responses in animals. In rats, SiO_2 NPs caused the depletion of dopamine and dopaminergic activity in the striatum (Wu et al. 2011). These outcomes are alarming because dopamine depletion in mice causes characteristic movement disorders of Parkinson's disease (Matsuura et al. 1997). Free ions released by some metal or metal oxide NPs also cause neurotoxicity. Exposure of CuO NPs to *Cyprinus carpio,* released free Cu^{2+} ions and inhibited the activity of cholinesterase, an important enzyme that hydrolyzes acetylcholine at cholinergic synapses (Zhao et al. 2011b). NPs can also induce morphological changes in the nerve cells of different parts of the brain and cause degeneration of nerve cells and may damage myelinated fibers too (Sharma 2007). Silver and copper NPs are more toxic than aluminum NPs, and the hippocampus is reported to be the most adversely affected by these NPs (Sharma et al. 2009a, b; Sharma and Sharma 2010). Intranasal instillation of TiO_2 NPs in the female mice for 30 days, caused morphological changes in neurons and decreased production of neurotransmitters in the sub-brain regions (Zhang et al. 2011). SiO_2 NPs administered by intranasal instillation to rats crossed the olfactory bulb to the striatum and induced oxidative damage and inflammatory responses (Wu et al. 2011). CNS toxicity by the NPs may occur by other mechanisms, like by destroying the phagocytic microglial cells and glioblastoma cells, but no such in vivo report is available.

In summary, NPs that enter the circulation can enter the CNS by crossing the BBB. In the brain, NPs induce inflammation and cell apoptosis and damage both neurons and glial cells. NPs alter the electrophysiological properties of neurons and release of neurotransmitters like dopamine from the neurons leading to behavioral disorders in animals.

Toxicity Assessment In the nervous system, NP toxicity assessments are focused on the drug delivery of solid lipid NPs (SLNs) in the brain. Radiography techniques like positron emission tomography and positron emission tomography/computed tomography system were used to assess the drug permeability across the BBB (Frigell et al. 2013). In rat, magnetic-labeled NPs were used as contrast agent for brain magnetic resonance imaging (Vera et al. 2014). After the injection of PEGylated silica NPs via the carotid artery, the transportation of NPs across the BBB was assayed by noninvasive in vivo imaging and ex vivo optical imaging (Liu et al. 2014). Silica NPs showed the potential role in the brain imaging. Confocal microscopy and electron microscopy studies were also used to assess the in vivo uptake of silica NPs by the brain cells (Liu et al. 2014; Tamba et al. 2018). Acute toxicity of the NPs to the brain has been visualized by brain histology examination (light microscopy and transmission electron microscopy) (Blasi et al. 2013) and fluorescence imaging (Vera et al. 2014).

Nervous injury is mainly assayed by changes in the behavioral and electrophysiological studies. In mature animals behavior observation is a gold standard for

evaluating perturbation of the CNS (Zhang et al. 2014). Behavioral studies with clinical signs of toxicity, like convulsions, tremors, salivation, nausea, etc., have been performed in well-established animal models (Pradhan et al. 2014). By examining the expression of genes related to spatial learning ability and memory function associated with the hippocampus toxicity of the NPs in nervous system has been studied in mice (Win-Shwe et al. 2008). Electrophysiological investigation with electroencephalogram bands (Horváth and Oszlanczi 2011) of the hemispheres of the brain and tail nerves is a convenient and reliable method to assess the toxicity of the cadmium NPs in the rat. Mitochondrion is the main cell organelle that utilizes the oxygen and is the main producer of reactive oxygen species. The brain has high oxidative metabolism (Akopova et al. 2014), and thus any damage to the cells of the brain will lead to alteration in the membrane potential of mitochondria and reactive oxygen species which may serve as marker of the nerve injury in the brain.

7.4.7 Nanotoxicity to the Reproductive System and Its Assessment

Nanotoxicity to the reproductive system is described as the detrimental effects of NPs on any stage of reproduction and pregnancy in the reproductive cycle, which may affect the fertility of males or females and development of healthy embryos in child-bearing age of the females (Adler et al. 2010). The toxic effects that impact the developing offspring at any stage of life before birth are called as developmental toxicity (Rogers and Kavlock 1998). NPs also have adverse effects on reproductive organ function, germ cells, and fertility of the animals.

Nanotoxicity to Female Reproductive Organs

Different studies on the toxicity of the NPs in female reproductive organs tend to focus on the uterus and ovaries. Smaller-sized NPs are more likely to accumulate in the uterus than larger NPs. Similarly, the solubility of the NPs also plays a major role in nanotoxicity. Intravenous administration of gold NPs in rats resulted in two fold higher accumulation of 1.4 nm size NPs at the uterine wall in comparison to the NPs of 18 nm or 80 nm size, administered at the same concentration (Semmler-Behnke et al. 2014). Iron oxide magnetic NPs (IOMNPs) of 10 nm size also accumulate more readily in the uterus of mice than NPs of larger size (Yang et al. 2015). Information on the toxicity of these NPs in the uterus is lacking. Gold NPs did not cause toxicity on the reproductive system when administered by an intraperitoneal route to either male or female mice (Chen et al. 2013). NPs also accumulate in other female reproductive organs, but there is a lack of overall clarity on their potential toxicity. Short-term oral administration of titanium dioxide (TiO_2) nanoparticles in rats increased total Ti level in the ovaries without general toxicity (Tassinari et al.

2014). Similarly, the single oral gavage of a high concentration (5 g/kg body weight) of TiO_2 NPs to adult mice produced no evidence of abnormal pathological changes in the ovaries over a period of 2 weeks (Wang et al. 2007). In contrast, intragastric treatment of TiO_2 NPs over 90 days resulted in ovarian damage in adult mice and altered the expression of genes associated with estrogen and progesterone synthesis and metabolism (Gao et al. 2012). High-dosage TiO_2 NP treatment altered the expression of genes related to apoptosis, inflammatory and immune responses, oxidative stress, cell proliferation, and ion transport (Gao et al. 2012). Thus, changes in sex hormone levels and damage to ovaries may have contributed in the decreased fertility and pregnancy rate observed in this study, in response to long-term exposure to TiO_2 NPs (Gao et al. 2012). Administration of nickel NPs (90 nm size) at the dose rate of 15 or 45 mg/kg body weight in adult rats decreased ovarian weight, increased apoptosis and infiltration of eosinophils and inflammatory cells into ovaries, and induced vascular dilation and congestion (Kong et al. 2014). Silver NP administration in mouse increased the in vivo expression of proinflammatory cytokines along with the loss of germ cells (Han et al. 2016). Estrogen and progesterone are main female sex hormones. There are few in vivo studies that suggest that the NPs can alter the expression of genes involved in the synthesis and metabolism of these hormones as mentioned above (Gao et al. 2012). Exposure of CdO NPs through inhalation route in mice resulted in ∼50% reduction 17β-estradiol level in serum, increased expression of estrogen receptors in the uterus, and decreased implantation rate of embryo (Blum et al. 2012).

Nanotoxicity to Male Reproductive Organs

NPs may also affect the male reproductive system as the small-sized NPs can easily cross the blood–testes barrier. The ability of NPs to cross the blood–testes barrier will determine their accumulation in the testes and thus their toxicity, if any. The impact of NPs may begin at the seminiferous tubules of the testes which reflects in the impaired spermatogenesis (Boisen et al. 2013). However, the impact of NPs on the male reproductive system varies from species to species (Gao et al. 2013). A review on NPs on spermatogenesis suggests precautionary measures in nanomedicines and understanding the passage of NPs through the blood–testes barrier (Yoshida et al. 2010). The toxicity of the NPs can begin from the fetal stage if the NPs are transmitted in utero or during pregnancy as described earlier. Exposing adult male mice to NPs can induce the changes in the seminiferous tubules and spermatogenesis directly. Intratracheal administration of carbon black NPs at high dosage (0.1 mg/mouse) for ten times every week resulted in histological changes in the seminiferous tubules and elevated serum testosterone levels (Bai et al. 2010). Some NPs are nontoxic to the testes; however, some NPs may induce toxic effects upon accumulation in the testes, with consequences for male fecundity (Brohi et al. 2017). Recent studies on AgNP ingestion by fruit flies suggested that AgNP accumulates in the testes and decreases the number of germline stem cells (Lafuente et al. 2016). Subchronic exposure of polyvinyl propylene (PVP)-coated Ag NPs

by oral administration altered the testicular histology and sperm morphology in rats (Hong et al. 2016). Exposure to TiO_2 NPs is also associated with reproductive toxicity in male animal models (Ritz et al. 2011).

Nanotoxicity to Embryo (Developmental Nanotoxicity)

Developmental toxicity of the NPs may be caused by transmission of NPs from the mother to the fetus through placenta. The small size and wide distribution of NPs to reproductive organs make them ideal candidates to breach the placental barrier. Rodent and zebrafish (*Danio rerio*) embryogenesis models have been used to explore the transplacental transmission of the NPs for in vivo studies. Such studies confirmed that NPs, like gold, TiO_2, SiO_2, carbon (C), and quantum dot NPs, can pass through the placental barrier (Takeda et al. 2009; Chu et al. 2010; Sumner et al. 2010; Refuerzo et al. 2011; Yamashita et al. 2011; Semmler-Behnke et al. 2014). Intravenous injection of Si and TiO_2 NPs in pregnant mice for 2 consecutive days resulted in 20–30% reduction in uterine weight, increased fetal resorption rate, and smaller fetuses at gestational days 16 and 17, resulting from placental dysfunction (Yamashita et al. 2011). Gold NPs of 5 nm size show higher transfer across placenta to the fetus, compared to 30 nm gold NPs after the intravenous injection, in Wistar rats (Yang et al. 2012).

In animal models, exposure to NPs during the prenatal period directly affects the brain and nervous system. Subcutaneous injection of TiO_2 NPs in pregnant mice resulted in the entry of NPs into the brain of the offspring, leading to blood vessel stenosis in the hippocampus and cerebral cortex (Takeda et al. 2009). A similar experiment in which TiO_2 (25–70 nm) was administered subcutaneously in the pregnant ICR mice, pups of the mice were having increased levels of dopamine (DA) and its metabolites in the prefrontal cortex and neostriatum (Takahashi et al. 2010). Intratracheal administration of diesel exhaust NPs to pregnant C57BL/6BomTac mice at 268 μg/animal induced changes in innate behavioral patterns of female offspring (Jackson et al. 2011). These studies suggested that the fetal brain tissue is highly vulnerable to NP toxicity.

NP toxicity affects fetal development and compromises fertility (Tsuchiya et al. 1996) and may cause changes in embryogenesis and anomalies in the fetal reproductive system. Subcutaneous injection of TiO_2 NPs in pregnant mice resulted in the entry of NPs to Leydig cells, Sertoli cells, and spermatids of the testis of male offspring aged 4 days and 6 weeks (Takeda et al. 2009). NP toxicity is also associated with abnormal fetal morphological development and organogenesis at different gestational periods (Tsuchiya et al. 1996). Oral administration of TiO_2 and platinum NPs in a high dose to pregnant dams causes significant increase in the fetal deformities and mortality (Philbrook et al. 2011; Park et al. 2010b). Oral administration of platinum (Pt) NPs in ICR mice, 14 days before and 4 days after mating, increased mortality of the pups, and decreased growth without deformities was noticed during the lactation period (Pietroiusti et al. 2011). Administration of SWCNTs to pregnant CD-1 mice, 5.5 days after implantation, also resulted in

skeletal defects, like divided cervical vertebra; reduced formation of new bones in the sternum, fingers, and toes; and phenotypic imperfections (Lim et al. 2011). Increased reactive oxygen species in both the placenta and fetus was associated with fetal malformations (Lim et al. 2011).

In summary, effects of NPs on reproduction have received much attention, while their effects on endocrine functions have been less well studied. NPs have the ability to accumulate in the reproductive organs of male and female. NPs cause less toxicity to the female reproductive organs as compared to male. In male NPs cross the blood–testes barrier and may disturb the spermatogenesis by producing lesions in the seminiferous tubules. NPs may alter the hormone levels of sex hormones in male and female. In pregnant animals, NPs less than 5 nm cross the placental barrier and may affect the development of the embryo. NP toxicity produces developmental effects in embryo, reduced body weight of the offspring and causes abnormalities in the reproductive organs of male.

Toxicity Assessment

NP toxicity assessment of the reproductive system is a relatively long-term study. Only a few researches (Hong et al. 2014; Kong et al. 2014; Di Bona et al. 2015; Roychoudhury et al. 2016; Hong et al. 2016) have focused on this kind of chronic study. However, it is an essential step for the clinical approval of NP products especially drugs. So far, various in vivo models have been developed to study nanotoxicity in different organs of this system (as reviewed by Saunders et al. 2015). Reproductive system is different for male and female and thus the strategies to assess the toxicity of NPs is different for female and male reproductive systems in some cases. Routinely for the assessment of nanotoxicity in male reproductive system, histopathology of the testes, sperm parameters, serum sexual hormones (e.g., testosterone), and the concentration of NPs in serum, spermatogonial stem cells, seminiferous tubules, and the testis are tested by electron microscopy (Li et al. 2016; Layali et al. 2016; Ren et al. 2016; Lafuente et al. 2016). For the assessment of nanotoxicity in the female reproductive system, sexual hormone (e.g., follicle-stimulating hormone, luteinizing hormone, progesterone, and estradiol) levels in the serum are measured (Saunders et al. 2015; Gao et al. 2012). Functions and histopathological analysis of major organs (ovary, uterus, and vaginal tract) are also evaluated to know the injury to the organ and concentration of the NPs accumulated (Semmler-Behnke et al. 2014; Yang et al. 2015). Specifically, organs such as testes, ovaries, uterus, and placenta (of parental or offspring generation) with potential resorption of NPs are suggested to be inspected (Semmler-Behnke et al. 2014; Yang et al. 2015). Electron microscopy can provide useful information about the histology and NP accumulation in the various tissues (Li et al. 2016; Ren et al. 2016; Lafuente et al., 2016). Reproductive index and offspring development need to be explored as some NPs penetrate the placenta–blood barrier and blood–testes barrier in rodents (Zhang et al. 2016; Yoshida et al. 2010; Brohi et al. 2017). Teratogenicity of NPs is of great concern on the fetus especially in case of prenatal exposure to the

NPs (Zhang et al. 2016; Lim et al. 2011). Also, the survival, growth, development, skeletal deformities, and reproduction of the offspring need to be mainly assessed upon prenatal exposure (Di Bona et al. 2015; Huang et al. 2014; Takeda et al. 2009; Takahashi et al. 2010).

7.4.8 Nanotoxicity to the Hematopoietic System

In adult mammals, the production and maturation of most blood cells occur within the bone marrow, whereas the lymphoid cells mature and activate in the spleen, thymus, and lymph nodes. The liver, thymus, and spleen may resume hematopoietic functions under some pathological conditions, causing enlargement of these organs. NPs may accumulate in the spleen and liver and cause toxicity. Toxicity to hematopoietic system may impair the production of blood cells by damaging hematopoietic stem cells or immune functioning, leading to severe diseases like leucopenia, thrombocytopenia, neutropenia, and anemia. NPs can accumulate in the bone marrow, as the bone marrow is an organ of the reticuloendothelial system. Among the various cells of innate immunity, accumulation efficiency of lipid NPs in monocytes (Peer et al. 2007) and macrophages (Sou et al. 2010) is higher than in lymphocytes, neutrophils, and dendritic cells. Many bone marrow-targeting drug delivery strategies using NPs with various surface chemistries have been developed (Sou et al. 2010; Leuschner et al. 2011).

Some NPs cause genotoxicity in the cells of the bone marrow. In rats, the oral administration of alumina NPs (30/40 nm) at doses of 1000 and 2000 mg/kg for 30 or 48 h resulted in the chromosomal aberrations in the bone marrow cells, indicating genotoxicity of alumina NPs to bone marrow (Balasubramanyam et al. 2009). Intraperitoneal injection of functionalized and pristine MWCNTs for 5 consecutive days to Swiss Webster mice resulted in a dose-dependent micronucleus formation and chromosomal aberrations in bone marrow cells and DNA damage in leukocytes (Patlolla et al. 2010). The adverse effects of NPs on hematopoiesis also depend on the route of administration. The inhalation of magnetic NPs (but not by intraperitoneal route) for 4 weeks in mice resulted in a decrease in the mean corpuscular volume and mean corpuscular hemoglobin content (indicators of impaired erythrocyte function) and decreased production of platelets and induced extramedullary hematopoiesis in the spleen, indicative of pathological conditions such as anemia (Kwon et al. 2009).

In summary, NPs entering the circulation through different routes may accumulate in the bone marrow, one of the primary organs of the reticuloendothelial system. NPs accumulated in the bone marrow causes genotoxicity to bone marrow cells. NP toxicity changes blood parameters, e.g., compromise erythrocyte functions, reduce the platelet count, and increase white blood cells count. Damage caused by the NPs to the bone marrow cells may induce extramedullary hematopoiesis in the spleen.

Toxicity Assessment The toxicity of NPs to hematopoietic system can be assessed by histological changes in major immune organs (e.g., spleen) which are preferably observed by H&E staining (Wang et al. 2016) (Fig. 7.2). TiO_2 NP exposure in the hairless mouse increased proliferation of the local macrophages in the spleen which was easily observed by the H&E staining of the tissue sections of the organ (Wu et al. 2009). Changes in the blood parameters like mean corpuscular volume, mean corpuscular hemoglobin, and number of WBCs and platelets also serve as indicator of nanotoxicity (Kwon et al. 2009). Histopathology of the bone marrow to find changes in the number of micronuclei formation may help in assessing nanotoxicity (Balasubramanyam et al. 2009; Patlolla et al. 2010). Transmission electron microscopy of spleen and liver can be used to study the toxicity of NPs to the hematopoietic system (Cho et al. 2009).

7.4.9 Conclusions

During the last few decades, advances in the field of nanotechnology has led to increasing number of applications of NPs in different areas like biomedicine, industry, drug delivery systems, cosmetics, food industry, and engineering and as contrast agent of imaging. Due to their wide applications, the possible toxicity of NPs cannot be neglected. Nanoparticles tend to be absorbed with different efficiencies from different exposure routes and get distributed to various organs where they may be partially degraded, excreted, or stably accumulated. Accumulation of NPs in different organ systems may cause damage either to the particular organ system or to the other organ systems. NP toxicity results in different types of lesions in different organs. In pulmonary toxicity NPs may cause inflammatory responses, granuloma formation, and fibrotic injury in the lung, or alteration in the fluid dynamics of the blood. In bone marrow toxicity, NPs may compromise erythrocyte functions, increase the number of WBCs, reduce the number of platelets, and induce extramedullary hematopoiesis in the spleen. NPs exposure to pregnant dams decreases the birth weight of offspring and causes damage to the reproductive systems of their male pups. NPs cause reversible damages to the male reproductive organs with minimal effect on the fertility, while accumulation of NPs in the mouse ovary disturbs the normal balance of sex hormones. In the digestive system, some NPs are quickly eliminated in feces, while remaining NPs may cause diarrhea, alter nutrient absorption, damage intestinal structure, and enter the circulation. In kidneys, the glomerular filtration barrier prevents large NPs (410 nm) from being excreted in urine, and smaller hydrophilic nanoparticles and some needle-like nanoparticles may bypass this barrier and enter the urine. The kidney is less sensitive to nanotoxicity, but copper NPs are known to cause renal toxicity. NPs may enter the CNS by damaging, penetrating, or bypassing the BBB. NPs may damage neurons and glial cells and affect nerve cell functions such as neurotransmitter release. The liver is one of the major organs where the NPs accumulate. Kupffer cells and hepatocytes retain NPs that alter the function of liver enzymes such as those in cytochrome P450

family. NPs have been shown to cause chromosomal aberrations and DNA damage in bone marrow cells. NPs can have significant effects across different systems and organs due to the close association and extensive intra- and intersystem communications between them. NPs can penetrate various biological barriers like dermal barrier, BBB, blood–testes barrier, and placental barrier.

Therefore, considerable attention should be paid to the biosafety assessments of NPs. The toxicity of the NPs to the different organ systems is assessed according to the properties of the NP and the type of organ system or tissue involved. Ultrastructural examination by electron microscopy, histopathological examination by staining tissue sections, analysis of serum parameters, enzyme function tests, analysis of different biomarkers, etc. also help in the nanotoxicity assessment. High-tech instruments like magnetic resonance imaging, positron emission tomography, and computed tomography can be used to assess the injury caused by NPs to some tissues.

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Chapter 8
Dendrimers as Drug Carriers for Cancer Therapy

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Abstract The efficacy of anticancer agents is often limited due to treatment-related toxicity, poor pharmacokinetics, and inadequate drug accumulation in the tumor. Advances made in the field of cancer nanomedicine have made it possible to reduce the toxicity, alter the pharmacokinetics and biodistribution, increase site-specific drug delivery, and enhance the efficacy of many therapeutic agents by using nanoparticles as drug carriers. These nanocarriers can be composed of polymers, lipids, proteins, or inorganic materials. Among these delivery systems, dendrimers form a separate class of branched polymer nanoparticles that has shown great promise in cancer drug delivery. In this chapter, we describe the application of dendrimers as nanocarriers for drug and gene delivery in cancer. We discuss the structures, properties, and various synthesis methods for dendrimers suitable for anticancer drug delivery. Further, we describe various types of dendrimers with appropriate examples in different therapeutic modalities of cancer. Recent examples of drug and gene delivery using dendrimers and their advantages are also presented. The application of tumor-targeted delivery systems using dendrimers is described. The chapter concludes with a description of current challenges with dendrimer-based drug delivery and efforts made to bring these promising systems to the forefront of cancer treatment.

Keywords Polyamidoamine dendrimer · Poly(propylene imine) · Poly-L-lysine · Cancer · Drug delivery · Gene delivery · Receptor targeting

8.1 Introduction

Cancer is the leading cause of disease-related deaths worldwide, and its incidence is increasing. Chemotherapy is among the most successful therapeutic modality for treating cancers of different stages. However, chemotherapy delivery presents several challenges, such as unfavorable pharmacokinetic profiles, low aqueous solubility, narrow therapeutic index, poor membrane permeability, rapid clearance, instability in circulation, and concerns over emergence of multidrug resistance (MDR) phenotypes in cancer (Ozols 2006; Fuertes et al. 2008). Side effects caused by the toxicity of chemotherapy drugs are also an unresolved clinical issue, mainly because of the lack of good delivery agents.

Similarly, gene therapy is another therapeutic modality that is gaining much attention due to its efficiency and increased tumor specificity compared with chemotherapy. This approach, however, requires specific delivery vehicles for successful application against cancer. The major challenge is achieving therapeutic concentrations at the tumor sites, since most gene therapy agents lack sufficient stability in the circulation. This lack of stability affects tumor-specific delivery. Therefore, delivery of therapeutic molecules, i.e., chemodrugs and gene molecules, into targeted tumor tissue is an important issue in cancer therapy (Bae and Park 2011).

The global drug delivery research community is currently focused on developing safe and targeted drug delivery strategies for cancer. To improve the biodistribution of cancer drugs, nanoparticles have been designed for optimal size and surface characteristics to increase their circulation time in the bloodstream. Numerous delivery methods have been developed. Among them, nanocarrier-based delivery systems have shown promising results (Singh and Lillard 2009). Nanocarriers are able to carry their loaded drugs selectively to cancer cells using the unique pathophysiology of tumors, such as their enhanced permeability and retention (EPR) effect and the tumor microenvironment (Maeda et al. 2000). Various metallic (gold and iron oxide)-, lipid (liposomes)-, and polymer-based nanocarriers have been studied for delivery of therapeutic molecules in vitro and in vivo (Bayda et al. 2017; Hu et al. 2017; Wilczewska et al. 2012). Some lipid- and polymer-based nanocarriers have been approved by the Food and Drug Administration (FDA) for clinical trials (Bobo et al. 2016). Although many of these nanoparticles are extensively explored in biomedical applications, their stability and toxicity are major issues. Metallic nanoparticles are prone to aggregation when they interact with biological molecules leading to rapid clearance. Lipid-based nanoparticles tend to burst release the encapsulated drugs due to their dynamic architecture, causing undesired distribution of the drugs and resulting in nonspecific toxicity.

Dendrimers are alternative carriers that can overcome the abovementioned limitations. These carriers form a special class of drug delivery systems that can be constructed with a well-defined molecular structure providing special opportunities for drug and gene delivery (Abbasi et al. 2014). Dendrimers are polymer-based, three-dimensional, highly branched monodispersed molecules that can be synthesized by sequential and precise introduction of unique branching structure. Ultimately, dendrimers are highly branched and have well-defined globular structures with enormous surface functionality (Klajnert and Bryszewska 2001).

The size of dendrimers is less than 100 nm. The use of dendrimers as nanocarriers for chemotherapy drug may provide significant advantages, including high drug loading, enhancement of water solubility, and low cytotoxicity; these versatile dendrimers have the ability to encapsulate both hydrophilic and hydrophobic molecules (Madaan et al. 2014; Choudhary et al. 2017). Another important advantage is that dendrimers can mediate the delivery of single-stranded or double-stranded, natural or synthetic DNA or RNA of any kind and any size (Chaplot and Rupenthal 2014). Dendrimers have increased overall ionic interaction with DNA compared with natural polyamines, polylysine, and liposomes, and they produce very stable and highly

soluble DNA complexes (Mendes et al. 2017). Dendritic polymers also have a broader concentration range between transfection and cytotoxicity. It has been demonstrated that some of these polymers increased the efficiency of plasmid-mediated gene transfer in vivo. Dendritic polymers have been recently studied in targeted drug, gene delivery, and imaging studies (Noriega-Luna et al. 2014).

8.2 Dendrimer Synthesis Strategies and Characterization

Molecular and polymer chemistry concepts are involved in the design of dendrimers. Dendrimer construction requires a step-by-step controlled synthesis (molecular chemistry) and the creation of a repetitive structure made of monomers (polymer chemistry). The synthesis process follows either divergent or convergent methods, as represented in Fig. 8.1. Dendrimers possess more symmetric, globular, and closed packed membrane structures with higher generations, whereas lower-generation dendrimers have an asymmetric and more open structure. The number of surface functional groups and size of dendrimers vary based on generation. The active surface functional groups (e.g., -COOH, -OH, -NH2, -SH) can also be synthesized with different core molecules. The generation of surface functional groups and internal cavities plays an important role in loading and conjugation of drug, gene, and targeting ligands.

8.2.1 Divergent Method

In the divergent method, the structure initiates from a multifunctional core and builds up one monomer layer. The first-generation dendrimer is then created by reacting the core molecule with monomers that have one reactive group per monomer as shown in Fig. 8.1. The sequence is then continued by activation of the inactive groups at the periphery and conjugation of another generation of monomer molecules, resulting in the second-generation dendrimer. This process is repeated for multiple layers; each layer represents one generation. Higher-generation dendrimers with larger molecular diameters are usually synthesized using this method.

PAMAM dendrimers that are widely used as nanocarriers are synthesized by the divergent method. Generally, these dendrimers are composed of an alkyl-diamine core and tertiary amine branches. Their terminal groups often end with different surface-active groups, such as -OH, -COOH, and -NH_2. The divergent method is also used to synthesize poly(propylene imine) (PPI) dendrimers with EDA and DAB as core groups, which are highly studied in biomedical applications. In this method, each step of product purification and degree of purity has limitation due to smaller molecular weight and size difference between desired dendrimer and imperfect dendrimers.

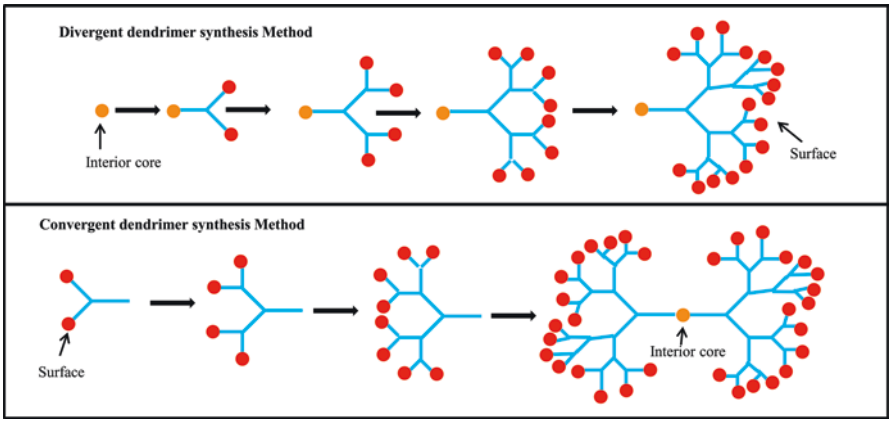

Fig. 8.1 Schematic representation of divergent and convergent dendrimer synthesis methods

8.2.2 Convergent Method

In the convergent method, dendrimer is synthesized layer by layer, but the core group terminates and the end groups react. These two (or more) peripheral branches react with a single joining unit that contains two (or more) active sites and one inactive site, as represented in Fig. 8.1. This reaction of two active sites repeats and joins with the peripheral branches. When the dendrons reach the target generation, they are then attached to a core molecule to yield the dendrimer (Xu et al. 1994; Grayson and Fréchet 2001). The molecular weight difference between desired dendrimer and its by-products is high, so it is easy to purify the required dendrimer from its by-products. Convergent method produces high-purity and homogeneity dendrimers because of lower generation and fewer reactive functional groups available. In a typical example, 5-aminolevulinic acid (ALA)-based dendrimers are synthesized by the convergent method by conjugating ALA residues to the periphery through ester linkages. Steric hindrance is generated when conjugating large dendron molecules with a small core molecule. Therefore, convergent method is useful for synthesizing lower-generation dendrimers and avoids steric hindrance.

The synthesized dendrimer structures can be characterized using different methods. The molecular weight and generation of dendrimer molecules can be identified using MALDI-TOF and ESI-MS. The chemical structure and functional groups can be identified by NMR, UV-visible, and FT-IR spectroscopy. The size and morphological properties of dendrimers can be measured by DLS analysis and TEM imaging, respectively. The internal structure of dendrimers can be confirmed with small-angle X-ray and neutron scattering and laser light scattering.

8.3 Chemistry and Structure of Dendrimers

Dendrimers are constructed with different generations; each generation increases the peripheral functional groups and cavity inside the dendrimers, as represented in Fig. 8.2. For biological applications, it is important to use biocompatible dendrimers. The most commonly used biocompatible dendrimer is polyamidoamine (PAMAM) dendrimer, which is available commercially. These PAMAM dendrimers are usually synthesized with an ammonia and ethylenediamine core. Ammonia has three branching units, while ethylenediamine has four branching units. These branching units are used to build up the different generations of dendrimers with the divergent approach, adding methyl acrylate to form amide bonds resulting from amine and ester reactions (Esfand and Tomalia 2001). The complete generations will result in amine surface functionality, whereas intermediate generations give carboxylate terminal groups to the dendrimers. The PAMAM dendrimers exist in 1–10 generations and with different surface functional groups (e.g., carboxylate, amine, alcoholic, sulfhydryl).

Another commercially available biocompatible dendrimer is poly(propylenimine) (PPI), synthesized from the butylenediamine (DAB) core molecule. The repetitive reaction is based on Michael addition of acrylonitrile to a primary amino group, followed by chemical reduction of nitrile groups into primary amino groups (Kaur et al. 2016). Another class of dendrimers based on poly-L-lysine (PLL) units, which have surface amine groups, has been explored as an antiangiogenic agent (Al-Jamal et al. 2010). PLL-based dendrimers are cationic dendrimers with amine surface groups with different generations. A PLL-based dendrimer-enhanced version of docetaxel (DTX; Taxotere®) called DEP™ docetaxel has entered Phase I clinical trials (Starpharma Holdings, Melbourne) in Australia. Starphama reported that in preclinical trials, DEP™ docetaxel showed significant tumor targeting and superior anticancer effects across a range of cancer types when compared with the clinically

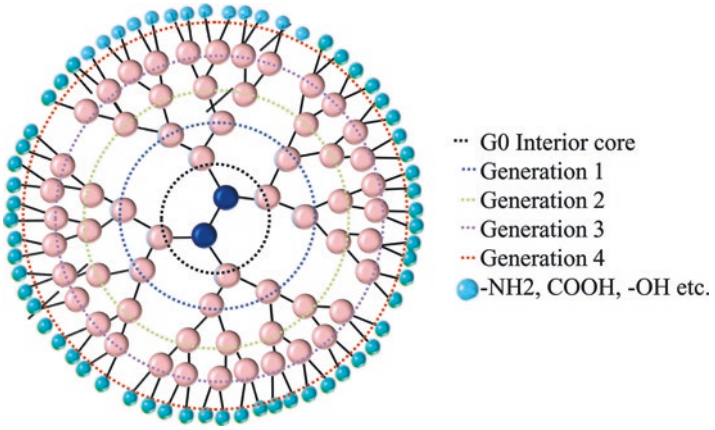

Fig. 8.2 General schematic representation of dendrimer structure. Each dotted line illustrates different generations. The chemical structures represent the surface groups of respective dendrimers

Table 8.1 Commercially available dendrimers and their surface functional groups

Dendrimer name	Surface functional group
Polyamidoamine	-OH, -COOH, -NH_2 C12 hydrophobe
Poly(propylen imine) tetramine dendrimer	-NH_2
Poly(ethylene glycol) linear dendrimer	Boc-protected amine
bis-MPA dendrimers	Acetylene, azide, Boc-protected amine, hydroxyl, carboxylic
Hyperbranched PEG dendrimers	Hydroxyl
PEG-core dendrimers	Hydroxyl, acetylene, ester
Phosphorous dendrimers	Dichlorophosphinothioyl
Poly-L-lysine	Amines
Poly(etherhydroxylamine)/poly(ester amine)	Amines, hydroxyl

approved drug, Taxotere®, a commercial Taxol formulation (Fox et al. 2009). Polyester dendrimers are another class of neutral surface charge biocompatible dendrimers that is mostly useful in drug delivery applications. It has low toxicity to normal tissues. Other chemical structures of peptide dendrimers, carbohydrate dendrimers, melamine dendrimers, and phosphorus dendrimers are also biocompatible and are being tested for various biomedical applications. Table 8.1 shows the commercially available dendrimers with various surface functional groups.

The structure and functional groups of dendrimers play a crucial role in drug and gene delivery applications. Water-soluble and biodegradable dendrimers are more biocompatible. Chemotherapeutic drugs can be functionalized with dendrimers through different methods, such as covalent conjugation, electrostatic interaction, and hydrogen bonding (Madaan et al. 2014). The surface functional groups of dendrimers (e.g., -COOH, -SH, -NH_2) can be used for direct conjugation with drugs through different chemical reactions (EDC/NHS or disulfide linkage) (Badalkhani-Khamseh et al. 2018). The drug molecules can also be conjugated with dendrimers using tumor microenvironment-sensitive linkages (hydrazone, disulfide) (Wang et al. 2016a, b). The carboxylate and amine groups possess negative and positive surface charges, respectively; those charges can be utilized to load drug molecules through electrostatic interaction.

Higher-generation dendrimers contain a cavity in their internal structure. These cavities can be utilized to load small molecules via coordinate bond or hydrogen bond formation (Choudhary et al. 2017). Nucleic acid molecules, such as plasmid DNA, siRNA, and shRNA, exhibit a negative surface charge due to their phosphate backbones. Thus, amine-functionalized dendrimers exhibiting positive surface charge can easily condense with negatively surface charged gene molecules, forming a compact complex that results in increased transfection efficiency (Palmerston et al. 2017). To increase the targeting efficiency, the dendrimers surface can be functionalized with proteins, aptamers, peptides, affibodies, and antibodies through chemical or physical interactions (Saad et al. 2008). To enhance the accumulation of the dendrimer nanoparticles in the tumor tissues, the dendrimers are usually modified with PEG molecules of different molecular weights. PEGylation prolongs the blood circulation, resulting in improved pharmacokinetics and biodistribution.

8.4 Biocompatible Dendrimers

The structural properties of size, charge, hydrophobicity, and functional groups are important parameters for in vitro and in vivo membrane and tissue interactions and biocompatibility. In vitro cationic dendrimers interact with anionic cell membranes and tissues, which causes toxicity by disturbing the cell membrane. The surface charge of dendrimers increases with increasing generations; lower-generation dendrimers are less toxic than are higher-generation dendrimers. With increasing concentration of dendrimers in the treatment, the toxicity increases (Duncan and Izzo 2005). Therefore, it is important to find the optimal tolerable concentrations of dendrimers when used in vivo.

Dendrimer toxicity is usually evaluated by hemolytic properties, membrane fluidics, in vitro cytotoxicity, and antibacterial activity. The surface modification of dendrimer with lipid molecules also results in toxicity, even at lower concentration (Albertazzi et al. 2013). Dendrimer toxicity can be reduced by choosing a biocompatible and biodegradable core and branched monomers and functionalizing active surface groups with biocompatible molecules such as PEG, amino acids, and carbohydrates.

Neutral and anionic dendrimers electrostatically cannot interact with biological tissues. Hence these dendrimers are optimal for clinical applications. An interesting study demonstrated that G4 PAMAM dendrimers show less toxicity and poor immune response and increase deeper tissue diffusion activity in the central nervous system (CNS) (Albertazzi et al. 2013). Another article reported that generation 4 and generation 8 viologen-phosphorus dendrimers are not toxic to B14 cells, but show cytotoxicity towards N2a cancer cells (Ciepluch et al. 2012). Polyester-based dendrimers degrade inside the body, which reduces the toxicity (Feliu et al. 2012). Disulfide linkage dendrimers also degrade in the intracellular environment due to the reducing nature of cells, resulting in negligible toxicity.

The *in vivo* toxicity of dendrimers resembles the *in vitro* toxicity. At lower concentrations, cationic PAMAM dendrimers shows minimal toxicity, while at higher concentrations, they cause liver toxicity. When the cationic groups are replaced with neutral polyethylene oxide, polyester dendrimers exhibit reduced *in vivo* toxicity (Jain et al. 2010).

8.5 Dendrimers for Drug Delivery

Dendrimers offer unique cavity-like structures inside their branches, enabling them to carry drugs by encapsulation. In addition, the presence of numerous peripheral functional groups in dendrimers can be utilized for conjugation of drugs. Dendrimers can carry hydrophilic drugs, hydrophobic drugs, or both (Nanjwade et al. 2009). The small size and globular architecture of drug-loaded dendrimers favor their easy permeability through vasculature pores and successful entry into the tumor milieu

for delivery of the drug payload. Thus, passively targeted conventional dendrimers use enhanced permeability and retention (EPR) effects for tumor-directed delivery of cancer drugs, which in turn reduces the exposure of normal tissues to drugs.

Paclitaxel is a hydrophobic anticancer drug. Delivery of this drug into the human body is a challenge, due to solubility and dispersibility. Dendrimers can be used as alternative carriers to deliver paclitaxel. Yang et al. utilized polyamidoamine-alkali blue (PTX-P-AB) dendrimer loaded with paclitaxel. Using pharmacokinetics, they found that PTX-P-AB dendrimer delivery increased lymphatic absorption and AUC values in lymph nodes compared with Taxol®. They concluded that PTX-P-AB was able to function as both lymphatic tracer and lymphatic targeting vector (Yang et al. 2016). Recently, another group reported that PTX conjugation was performed through enzyme-sensitive linker glycylphenylalanylleucylglycine tetra-peptide by an efficient click reaction to PEGylated peptide. This enzyme-sensitive PTX delivery increases cytotoxicity against 4 T1 cancer cells while reducing dendrimer toxicity to normal cells when compared with free PTX. In vivo studies also supported extended circulation time in tumors, showing therapeutic effects in the 4 T1 breast cancer model (Li et al. 2017).

Targeted dendrimer nanocarriers have been developed for tumor-specific delivery of drugs by modifying the dendrimers with ligands that have specific affinity toward certain cancer cell surface receptors. Anchoring poly(propylene imine) dendrimers with folate, dextran, or galactose resulted in targeted delivery of anticancer drug paclitaxel (PTX) in HeLa and SiHa cells (Kesharwani et al. 2011). However, the therapeutic efficacy of PTX was different for folate-, dextran-, and galactose-modified dendrimer formulations (IC50 values of 0.05, 0.2, and 0.8 μM, respectively). They showed that the folate-anchored dendrimer-PTX formulations had the highest targeting potential. All of these formulations showed higher cell-killing efficiency than did free PTX.

Another study demonstrated that LFC131 peptide functionalized PAMAM dendrimers encapsulated with common anticancer drug doxorubicin (Dox). Researchers studied CXCR4 receptor targeting in breast cancer cells and observed significantly increased therapeutic efficiency with targeted dendrimers over nontargeted dendrimers. Further, the targeted LFC131-PAMAM reduced migration of BT-549-Luc breast cancer cells toward chemoattractant (Chittasupho et al. 2017). In another report, peptide-based capsid-like mimic dendrimers increased tumor penetration and drug accumulation in solid tumor tissue. These Dox-loaded delivery systems facilitate capsid-like components, nanostructures, and pH-responsive controlled drug release. Capsid-like structures increased the accumulation of Dox and in vitro and in vivo therapeutic effects in 4 T1 tumor-bearing BALB/c mice while reducing toxicity (Li et al. 2016).

Dendrimer nanoparticle delivery of other classes of anticancer drugs, such as cisplatin and 5-fluorouracil (5-FU), has also been studied (Tran et al. 2013). Cisplatin and 5-FU were loaded onto neutral surface PEGylated polyamidoamine (PAMAM) dendrimer (G 3.0) and negatively charged carboxylated PAMAM dendrimer (G 2.5). The formulations of cisplatin and 5-FU showed therapeutic activity

against NCI-H460 lung cancer and MCF-7 breast cancer cell lines, respectively (Tran et al. 2013).

In a recent report, ursolic acid (UA), a natural triterpene acid, was used for suppression of tumor growth and metastasis. However, bioavailability was low due to hydrophobicity. To increase UA dispersibility, a low-polyamidoamine (low-PAMAM) dendrimer-based formulation was developed through self-assembly. This dendrimer-UA complex enhanced cytotoxicity, attenuated the migration and adhesion of SMMC7721 liver cancer cells, and suppressed metastasis. Moreover, in vivo study revealed an improvement in blood circulation time for the dendrimer-UA complex that ultimately resulted in tumor growth inhibition in a mouse model (Shen et al. 2018).

Camptothecin (CPT), a poorly water-soluble plant alkaloid isolated from *Camptotheca acuminata*, is widely used as a cancer treatment. Cheng et al. reported that PAMAM dendrimer-CPT complexes could enhance the aqueous solubility, a major issue during drug formulation, of CPT in clinical trials (Cheng et al. 2008). In another ester-linked glycine and β-alanine spacers conjugated G3.5 PAMAM dendrimer-SN38 (7-ethyl-10-hydroxycamptothecin) was used against colorectal cancer metastases. This G3.5-SN38 conjugate has advantages in that the drug is covalently conjugated to the dendrimer rather than complexed, which increases stability in gastric and intestinal environments and reduces uncontrolled release. Further, CPT delivery in HT-29 cells using G3.5-glycine-SN38 and G3.5-βAlanine-SN38 formulations showed IC50 concentrations of 0.60 and 3.59 μM, respectively (Goldberg et al. 2011).

8.6 Dendrimers for Gene Delivery

RNA interference (RNAi) is a process in which a desired gene is silenced or part of its expression is knocked down through a complementary RNA introduced into the cell. RNAi is a conserved biological process among multicellular organisms, in which double-stranded RNA is processed by the enzyme dicer into ≈21–23 bp double-stranded fragments known as small interfering RNAs, or siRNAs (Carthew and Sontheimer 2009). This process forms an "RNA-induced silencing complex" (RISC), which scans mRNAs for homology and, upon sequence-specific binding, promotes the destruction of target mRNAs through enzymatic activity integrated in the complex (Tijsterman and Plasterk 2004). In cancer, therapeutic RNAi is moderated by introducing small interfering RNA (siRNA), short hairpin RNA (shRNA), or microRNA (miRNA) complementary to the target mRNA in cancer cells. The siRNA are known to be highly specific in inactivating the targeted gene. Thus, they can act as therapeutics or can inactivate genes that can enhance the activity of a co-anticancer agent, such as a small molecule inhibitor or a chemotherapeutic. RNAi agents are vulnerable to enzymatic digestion and rapid removal from the circulation upon systemic administration.

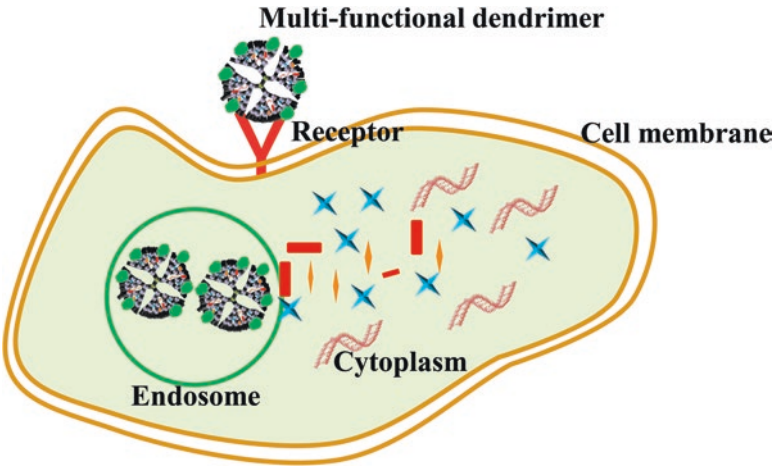

Fig. 8.3 Schematic illustration of multifunctional dendrimer uptake by cells through receptor-mediated endocytosis and entry into endosomes. After endosomal disruption, therapeutic and diagnostic molecules enter the cytoplasm

Dendrimers, due to their unique structure and cationic functionality, can readily complex with and condense nucleic acid therapeutics. They can carry RNAi agents, protect them from enzymatic digestion, and prolong the circulation time. In addition, dendrimers allow various surface modifications for targeted delivery of RNAi agents (Tambe et al. 2017). Different dendritic structures, such as polyamidoamine (PAMAM), poly(propylene imine) (PPI), poly-L-lysine, poly(etherhydroxylamine) (PEHAM), poly(ester amine) (PEA), and polyglycerol, have been used as gene delivery systems (Table 8.2). Among various dendrimers, PAMAM has been widely considered an efficient carrier for gene transfer and, recently, for RNAi delivery (Yang et al. 2015a, b). Figure 8.3 illustrates the uptake of multifunctional dendrimers by cells and the entrance of the therapeutics and image agents into the cytoplasm via endosomal escape.

The nucleic acid to dendrimer charge ratio, nitrogen to phosphate ratio (N:P ratio), and heat activation of the dendrimer are important parameters that determine dendrimer-nucleic acid complexation and efficient transfection. In a typical example, PAMAM (G4, G5) dendrimers were complexed with plasmid DNA, and their ability to transfect cells in vitro and in vivo was evaluated (Navarro and Tros de Ilarduya 2009). Compared with naked DNA, these PAMAM dendrimers were more effective in protecting DNA from DNase and transfection efficiency. The N:P ratio of 10:1 between dendrimer and DNA was optimal in inducing efficient transfection. Moreover, heat-activated dendrimer showed enhanced efficiency in transfection compared with nonactivated or intact dendrimer-DNA complexes. In vivo studies demonstrated that intravenously administered G4 and G5 heat-activated dendrimer-DNA complexes showed increased gene transfection efficiency compared with nonactivated complexes (Navarro and Tros de Ilarduya 2009).

Table 8.2 Example of dendrimers utilized for drug and gene delivery

Dendrimers	Therapeutic agent	References
Polyamidoamine	Paclitaxel	Yang et al. (2016) and Li et al. (2017)
Poly(propylene imine)	Paclitaxel	Kesharwani et al. (2011)
Polyamidoamine	Doxorubicin	Chittasupho et al. (2017)
Peptide-based capsid-like mimic dendrimers	Doxorubicin	Li et al. (2016)
PEGylated and carboxylate polyamidoamine (PAMAM)	Cisplatin and 5-fluorouracil (5-FU)	Tran et al. (2013)
Polyamidoamine	Ursolic acid (UA)	Shen et al. (2018)
Polyamidoamine	Camptothecin (CPT)	Cheng et al. (2008) and Goldberg et al. (2011)
Polyamidoamine	Plasmid DNA	Navarro and Tros de Ilarduya (2009) and Huang et al. (2007)
Amine-terminated PAMAM	siRNA	Jensen et al. (2011) and Shen et al. (2014)
PAMAM G4.0-PEG-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	siRNA	Biswas et al. (2013)
Poly(propylene imine)	siRNA	Tietze et al. (2017)

Similarly, siRNA delivery using dendrimers requires optimization of key parameters, such as the N:P ratio between dendrimer and siRNA and the generation of dendrimer used for complexation with siRNA. Jensen et al. studied various dendrimer generations to obtain optimal complexation efficiency with siRNA. They observed that dendrimer generations with low charge density (e.g., G1) lacked siRNA condensation ability. Higher efficiency in dendriplex (dendrimer-siRNA complex) formation was observed with G4 and G7 dendrimers with high charge densities. Among the dendrimer generations studied, flexible G1 and rigid G7 dendrimers displayed unfavorable thermodynamic properties. The researchers concluded that G4 dendrimer showed better dendriplex formation ability than did other dendrimers used in siRNA encapsulation (Jensen et al. 2011).

The N:P ratio plays a crucial role in forming complex and release kinetics. Although a dendrimer-siRNA complex with an appropriate N:P ratio exhibits therapeutic efficacy, it may cause toxicity. To improve safety, dendrimer-siRNA complexes can be coated with liposomes. These liposome-coated complexes are called dendrosomes. Dutta et al. reported successful delivery of siRNA targeted to E6 and E7 oncogenes in cervical cancer cells using a novel dendrosome nanocarrier DF3. In the first step, a dendrimer-siRNA complex (viz., 4D100) was optimized for transfection efficiency in cells. 4D100 was toxic to cells, but when it was encapsulated in liposomes to form dendrosomes (DF3), the toxicity was negligible. Compared with other formulations tested in their study, DF3-containing siRNAs showed considerable knockdown of the target genes (E6 and E7) in cervical cancer cells (Dutta et al. 2010).

PEGylation into dendrimers increased the transfection efficiency. Shen et al. studied G5.0 and G7.0 PAMAM dendrimers conjugated with PEG 5000 Da for the

delivery of syndecan-4-specific siRNA and caveolin-1 protein in C2C12 mouse myoblasts and the HepG2 human hepatocellular carcinoma cell line. PEGylation was increased to give the polyplexes higher syndecan-4 siRNA transfection efficiency with low immune-recognition response and cytotoxicity. The results showed a significant improvement in the cellular uptake of PEG-PAMAM dendrimer polyplexes in HepG2 with the downregulation of syndecan-4 and upregulation of caveolin-1 (Shen et al. 2014).

In another study, DOPE lipid was utilized for conjugation along with the PEG molecule. A triblock copolymeric system composed of PAMAM G4.0-PEG-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PAMAM-D-PEG-2K-DOPE) was synthesized and condensed with siRNA. The hydrophobicity of DOPE allows cellular interaction for enhanced cell penetration and to achieve increased siRNA condensation. The PAMAM-D-PEG-2K-DOPE micellar nanocarrier formed stable complexes with siRNA with serum stability and increased cellular uptake of siRNA, leading to better target gene knock-down when compared with the PAMAM G4.0 dendrimer. Further, PAMAM G4.0-D-PEG-2K-DOPE/PEG-5K-PE micelles showed potential for drug/siRNA codelivery (Biswas et al. 2013).

In a different study, to improve siRNA therapeutic efficiency, single-chain fragment variables (scFvs) were conjugated to poly(propylene imine) dendrimers and functionalized with maltose (mal-PPI) for siRNA delivery. Using biotin–neutravidin bridging, researchers conjugated mal-PPI with epidermal growth factor receptor variant III (EGFRvIII), monobiotinylated anti-EGFRvIII scFv fused to a *Propionibacterium shermanii* transcarboxylase-derived biotinylation acceptor (P-BAP). Compared with the control polyplex with nonspecific scFv-P-BAP, the polyplex with EGFRvIII scFv delivered siRNA exclusively toward tumor cells by receptor-mediated endocytosis. The authors concluded that the use of EGFRvIII scFv-modified mal-PPI-based polyplexes is an effective strategy for tumor targeted delivery of siRNAs (Tietze et al. 2017) (Table 8.2).

8.7 Dendrimers for Receptor-Targeted Delivery

Cancer cells often overexpress specific receptors. By exploiting the receptor–ligand affinity, drug delivery systems can be modified using specific ligands for those receptors to achieve cancer cell-targeted drug delivery. Receptor-targeted drug delivery has been extensively explored for active targeting. However, active targeting is achieved with a carrier surface functionalized with active targeting ligands that have high binding affinity towards a specific cell type, tissue, or organ. Some targeted delivery systems are currently in clinical trials (Vhora et al. 2014). To improve the specificity of dendrimer nanocarrier systems, ligands specific to cancer cell receptors can be conjugated. Commonly used ligands include transferrin, folic acid, peptides (e.g., Arg-Gly-ASP), and aptamers.

Transferrin (Tf) is an iron-chelator protein and has affinity toward transferrin receptors (Tf-R), which are overexpressed by many cancers. This ability of cancer cells allows them to internalize Tf in high levels compared with levels in normal

cells. Tf-conjugated targeted dendrimer drug delivery systems exploit these high Tf levels. A typical example of Tf-ligand-based targeted dendrimer drug delivery was reported by Huang et al. (2007). They constructed a high-branching nanoscopic PAMAM dendrimer conjugated with Tf using a bifunctional polyethylene glycol linker and tested the uptake in brain capillary endothelial cells (BCECs) and in Balb-C mouse brains. The transfection efficiency of the PAMAM-PEG-Tf/DNA complex was much higher than that of PAMAM/DNA and PAMAM-PEG/DNA complexes in BCECs. The luciferase activity obtained from DNA complexed with PAMAM/Tf complex was 2.25-fold higher than that from DNA complexed with PAMAM in mouse brains after IV administration. At the 10:1 weight ratio of PAMAM/DNA, Tf gene expression for the PAMAM-PEG-Tf/DNA complex was approximately twofold higher than that of the PAMAM/DNA and PAMAM-PEG/DNA complexes in the brain (Huang et al. 2007).

Integrins (e.g., integrin alpha-v beta-3) are overexpressed in activated endothelial cells, newborn vessels, and some tumor cells, but are not present in resting endothelial cells and most normal organ systems. Arg-Gly-Asp (RGD) peptide specific to integrins has also been explored for targeted dendrimer-based drug delivery toward tumor neovasculature and tumor cells. Kong et al. studied the use of cyclic-RGD peptide c(RGDfK) ligand for targeted delivery of PAMAM by modifying PAMAM G4.0–25% C12 with fluorescein isothiocyanate. RGD-modified PAMAM showed significantly higher cellular uptake than did non-RGD-modified PAMAM, as confirmed by fluorescence microscopy assay with 22RV1 cells. PEGylation successfully reduced the toxicity of PAMAM in 22RV1 cells with high expression of integrin alpha-v beta-3. No apparent toxicity was observed with the modification with c(RGDfK). Drug release was observed in targeted tumor sites, and the therapeutic efficiency of 10-hydroxycamptothecin was enhanced with RGD-modified dendrimer delivery in 22RV1 cells and MCF-7 cells compared with their non-RGD counterparts (Kong et al. 2014).

Folic acid is a well-known ligand for selective targeting of drugs into folate receptor-positive tumor cells. Conjugating folic acid molecules to dendrimers allows them to target tumor cells with folate receptor expression to enhance the therapeutic efficiency of the drug. Jain et al. (2014) reported the use of FA-conjugated poly-L-lysine (PLL) dendrimers (FPLL) to which the water-soluble drug doxorubicin (Dox) was conjugated through a pH-sensitive linker. FPLL showed significant antiangiogenic activity in human umbilical vein endothelial cells (HUVEC), compared with nontargeted dendrimer. When compared with free Dox, the FPLL formulation showed higher levels of accumulation in MCF7 xenografts in a mouse model and enhanced therapeutic activity, leading to significantly prolonged survival (Jain et al. 2014).

FA-conjugated dendrimers are also under study for the construction of multifunctional architectures for targeted cancer drug delivery and as diagnostic tools. Two or more functional end groups can be introduced to dendrimers to allow conjugation with multiple agents, such as drugs, genes, fluorescent dyes, MRI agents, and targeting ligands (Fig. 8.4). A recent study demonstrated the use of folic acid (FA)-conjugated PAMAM G5.0 dendrimers as a platform for constructing a

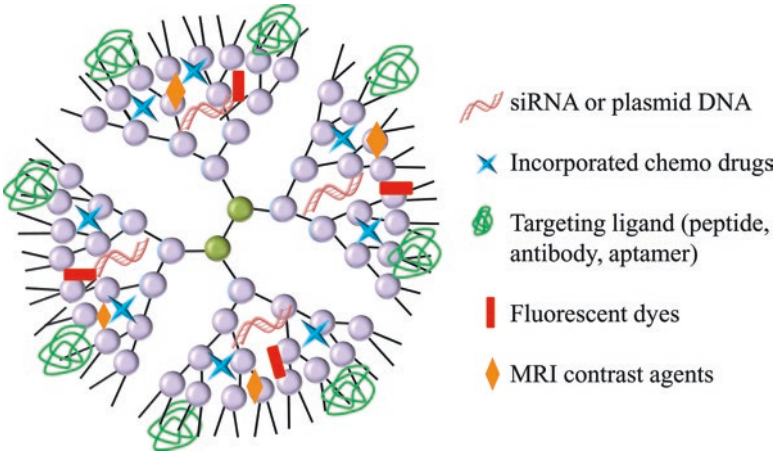

Fig. 8.4 Schematic representation of multifunctional dendrimer nanoparticles functionalized with therapeutic molecules (drugs and genes), diagnostic agents (fluorescent dyes and MRI agents), and targeting ligands

multifunctional theranostic system for targeted cancer imaging and therapy (Zhu et al. 2014). PAMAM-entrapped gold nanoparticles were covalently conjugated with fluorescein isothiocyanate, PEG-modified-a tocopheryl succinate, and PEGylated folic acid (Au-TOS-FA-DENPs). In vitro cellular uptake assay and flow cytometric study with U87MG and L929 cells showed that the conjugate could be specifically delivered to cancer cells that overexpressed folic acid receptors via receptor-mediated binding and endocytosis. In CT imaging, the CT value of U87MG with high expression of folic acid was significantly enhanced with Au-TOS-FA-DENPs. Further, a significant decrease in U87MG-HFAR cell viability was observed when treated with Au-TOS-FA-DENPs compared with the nontargeted counterpart. An in vivo study reported no toxic side effects and obvious tumor inhibition in BALB/c nude mice (Zhu et al. 2014).

Aptamers are an attractive class of ligands that exhibit many desirable properties for constructing a targeted drug delivery system. These ligands are short, single-stranded oligonucleotides obtained through the process of systematic evolution of ligands by exponential enrichment (SELEX). These oligonucleotides are known for their high binding affinity and target specificity, low immunogenicity, and versatile synthetic accessibility. Aptamer–dendrimer bioconjugates have been created for targeted delivery of chemotherapeutic drugs and gene silencing agents for cancer therapy. Recently, one report demonstrated that AS1411 aptamer functionalized on PAMAM dendrimer, which conjugated with 10-bromodecanoic acid (10C) and 10C-PEG. These aptamer-functionalized dendrimers targeted nucleolin ligand and specifically knocked down Bcl-xL protein with shRNA plasmid delivery. The aptamer-modified dendrimer significantly improved the transfection efficiency when compared with the nontargeted dendrimer in A549 cells. This improved transfection efficiency led to increase gene silencing and apoptosis (Ayatollahi et al. 2017).

MicroRNA delivery has been explored using aptamer conjugated-dendrimer systems. In a recent study, micro-RNA-34a (miR-34a), a potent endogenous tumor suppressor in NSCLC, was encapsulated into S6 aptamer-conjugated dendrimer to form a lung cancer-targeted gene delivery system (PAM-Ap/pMiR-34a NPs). The aptamer conjugation to PAM significantly improved the pMiR-34a cellular uptake and transfection efficiency in NSCLC cells. They showed that PAM-Ap/pMiR-34a NPs enhanced the regulation of targeted genes BCL-2 and p53 in vitro. PAM-Ap/pMiR-34a NPs significantly inhibited the cell growth, migration, and invasion and induced significant apoptosis of lung cancer cells compared with nontargeted NPs (Wang et al. 2015).

Recently, we demonstrated that FA-conjugated PAMAM dendrimer can be successfully used for folate receptor alpha (FRA)-targeted combinatorial delivery of CDDP and HuR siRNA for lung cancer therapy. Here, CDDP and HuR siRNA were encapsulated through a hydrolysis method and electrostatic interactions, respectively. Further, folic acid was conjugated to the surface of dendrimer functional groups for FRA-targeted delivery. The combinatorial delivery showed significant enhancement in therapeutic activity of CDDP and HuR siRNA in nonsmall cell lung cancer cell lines (H1299 and A549). Normal lung fibroblast cells (MRC9) that had low FRA expression levels did not show significant toxicity with our formulation. The FRA-targeted combined delivery also showed significantly higher therapeutic efficiency of CDDP and HuR siRNA than did nontargeted delivery (Amreddy et al. 2017).

Despite these promising outcomes, there are some challenges in using targeted dendrimers for drug delivery. First, a clear understanding of the differential expression and accessibility of cell surface receptors in the target cancer is required. Second, the ligand density in the dendrimer should be optimal for efficient interaction with the cell surface receptors. The proper choice of ligands and ideal conjugation chemistry will increase the target specificity. For example, aptamers should be carefully designed to avoid any multimerization interactions with drugs, as maintaining proper confirmation of aptamers after conjugation with dendrimers is crucial in determining the receptor specificity. Another important step is the choice of conjugation chemistry between ligands and the dendrimer host that should control the stability and release of ligand appropriately in the in vivo environment.

8.8 Dendrimer–Light Interaction Therapies

8.8.1 Photodynamic Therapy

Photodynamic therapy (PDT) is a type of phototherapy that kills bacteria, fungi, and viruses. It is used in treatment of skin, head, and neck, lung, and bladder cancers. PDT involves three components: photosensitizer (PS, a chemical substance), light, and reactive oxygen species (ROS). The PS is administered into the desired tissue and followed by irradiation with NIR light with the specific wavelength of excitation of the PDT agent (PS). Irradiation will cause generation of ROS in the presence

of molecular oxygen. This kills cancer cells, as ROS damages the cell membrane and organelles, resulting in cell death. Most potential PS molecules are not solubilized in aqueous media; when administered into the body, PS molecules interact with normal tissues resulting in nonspecific toxicity. The targeted delivery of PS molecules which can be achieved through nanocarrier-based delivery is an alternative. Dendrimers are one of the best carriers of PS molecules.

PS molecules can be encapsulated into dendrimers by methods such as electrostatic, physical interaction, and covalent conjugation. In a typical example, the anionic and cationic phosphorus dendrimer were used to encapsulate methylene blue and rose bengal (RB) PSs through electrostatic and π–π interactions, respectively. These polyanionic and polycationic combinations resulted in better stability and therapeutic effect, and identified to be better PDT carriers (Dabrzalska et al. 2015). Encapsulation of PS by physical interaction can occur in organic and aqueous solvent mixtures at different ratios. The RB PS physically interacts with PAMAM dendrimer when incubated in a mixture of methanol and water. The resulting PAMAM–RB dendrimer showed improved phototoxicity in mouse lymphoma cell lines (Karthikeyan et al. 2011). The photosensitizer chlorin e6 (Ce6) covalently conjugated to G4.5 PAMAM dendrimers showed enhanced photodynamic therapeutic effect than did free Ce6 (Bastien et al. 2015). Another approach implemented a PAMAM dendrimer-PS complex in image-guided PDT (Yang et al. 2015a, b). In this approach, the Ce6 PS-conjugated polyethyleneimine-PEGylated ceria nanoparticles increased solubility and stability of Ce6 PS in an aqueous environment. Furthermore, the enhanced cellular uptake and therapeutic effect of Ce6 was observed by image-guided therapy approach.

Receptor-targeted PS delivery also improves therapeutic effect in cancer treatment and reduces the toxicity to normal tissues. A study reported that HER2 peptide and 5,10,15,20-tetrakis(4-hydroxyphenyl)-21H,23H-porphine (PS) were covalently conjugated to the PAMAM dendrimer. This HER2 receptor-targeted PS delivery increases cell uptake and PDT effects in HER2 overexpressed SKOV3 ovarian cancer cells in vitro and in vivo compared with when administered in HER2-negative expressing MCF7 cell line (Narsireddy et al. 2015).

The PEGylation of dendrimers improves dispersibility and prolong circulation that improves cytotoxicity. The PEGylation of PAMAM and PPI dendrimers also increases protoporphyrin-IX stability in physiological conditions resulting in higher cytotoxicity in the desired tissue (Kojima et al. 2007). Dendrimers in other forms of complex called micelles also increase the PDT effects. The polyanion dendrimer porphyrins (DPs) complexed in other micelles composed with PEG-b-P(Asp) effectively delivers PS, leading to enhanced PDT efficacy (Stapert et al. 2000).

8.8.2 Photothermal Therapy

Photothermal therapy (PTT) involves a photo-activating agent that will generate heat upon light irradiation to kill cancer cells. Unlike PDT, PTT does not generate singlet oxygen. Normal tissues can bear up to 42 °C heat, whereas cancer tissues

cannot tolerate this temperature. Some of the nanoparticles are inherent to act as photothermal agents. Metallic-based gold nanorods, gold nanoshells, and graphene oxide materials are the typical examples for photothermal agents. For effective delivery and treatment, these nanoparticles can be combined with polymer-based nanoparticles. Gold nanorods absorb the radiation in the near-infrared region, whereas gold nanoparticles absorb at the visible region. Hence, nanorods show better therapeutic efficiency in photothermal therapy. The dendrimer-stabilized gold nanorods show better photothermal effects than do dendrimer-stabilized gold nanoparticles.

The safety of photothermal agent development is also an important parameter in terms of clearance from the body, which requires photothermal agents small in size and effectively efficient. Wang et al. developed ultrasmall-Au DSAuNRs, which showed better safety and higher photothermal affect in vivo (Wang et al. 2016a, b). Photothermal ablation can also be utilized to deliver other therapeutic molecules (genes and drugs), to further increase the controlled therapeutic effect. Zhang et al. described PAMAM dendrimers grown onto mesoporous silica-coated gold nanorods via a divergent method. The dendrimers were loaded with siRNA and Dox therapeutics. Upon NIR light irradiation, siRNA and Dox were released along with the PTT effect, resulting in multitherapeutic effects in cancer cells and reduced toxicity to normal cell lines (Zhang et al. 2017). The CTAB is known to be toxic in higher concentrations; replacing CTAB surfactant combined with dendrimer reduces the toxicity to normal tissues. Li et al. synthesized gold nanorods by CTAB and replaced the CTAB with PAMAM dendrimer, followed by conjugation with arginine-glycine-aspartic acid (RGD) peptides for targeted delivery. They reported selective photothermal effects in the $\alpha_V\beta_3$ overexpressing A375 cell line compared with MCF7 cell lines expressing less $\alpha_V\beta_3$ (Li et al. 2010).

8.8.3 Boron Neutron Capture Therapy

Neutron capture therapy is a noninvasive therapy used for localized tumor treatments. It is mainly useful for brain and head and neck tumors. The mechanism involves a two-step process. First, nontoxic neutron-capturing agents are administered into tumor. Usually, boron-10 (^{10}B) and gadolinium-157, 155 ($^{157, 155}$ Gd) metals act as neutron-capturing agents. This step is followed by irradiation with a neutron beam on targeted tissue. Then those metal substances convert into radioactive substances that decay helium and energy as by products. The energy thus generated helps to kill the cancer cells. For effective administration of ^{10}B into targeted tissue, nanocarrier systems are useful tools (Laramore et al. 1994; Aromando et al. 2009).

Polymer-based dendrimer nanoparticles are also used in boron neutron capture therapy for cancer. Different generations of PAMAM dendrimers are utilized for ^{10}B delivery. The ^{10}B molecules can be conjugated with PAMAM dendrimers via different chemical methods. Barth et al. reported that the boronated molecule isocyanato

polyhedral borane [Na(CH3)3NB10H8NCO] was conjugated to second and fourth generation of amine-terminated PAMAM dendrimers (Barth et al. 1994). In further studies, boronated dendrimers were conjugated with SPDP-derivatized MoAb to improve the tumor accumulation in the therapeutic range. Another report demonstrated that fifth-generation PAMAM dendrimer was conjugated with boronated molecules and chimeric MoAb cetuximab, which directs to EGFR- and EGFRvIII-expressed brain tumors (Wu et al. 2004).

PEG conjugations on dendrimers will effect pharmacodynamic properties. Receptor-targeted boron delivery further increases the boron accumulation and BNCT effect and reduces the uptake by reticuloendothelial system. Shukla et al. conjugated boronated poly(ethylene glycol) (PEG) with third-generation PAMAM dendrimers. The PEGylated boronated complexes showed more tumor uptake than did the corresponding non-PEGylated dendrimers (Shukla et al. 2003).

8.9 Dendrimers in Imaging and Diagnosis

Dendrimer molecules do not have inherent diagnostic and imaging properties. To create an imaging and diagnostic tool with dendrimer molecules, dendrimers are externally loaded or encapsulated with imaging and diagnostic agents. Usually, gadolinium (Gd III) and superparamagnetic iron oxide (SPIO) nanoparticles provide magnetic resonance imaging (MRI) contrast. The loading of these agents is useful for tracking dendrimers inside the body. Gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA) and gadolinium-tetraxetan (Gd-DOTA) chelates conjugated with different generations of dendrimers increase MRI signal intensity (Zhu et al. 2008; Rudovský et al. 2006). Higher-generation dendrimers will give better MRI contrast than will lower-generation dendrimers, since more metal chelates can be conjugated to higher-generation dendrimers.

Another important imaging technique is X-ray computed tomography (CT). Iodinated and gold nanoparticle-based agents are being used in CT imaging (Cormode et al. 2014). In a typical example, different generations of G3, G4, and G5 dendrimers with amine surface groups were conjugated with tri-iodophthalamide agents. This approach showed improved in vivo imaging with intravascular enhancement and a half-life of 35 min with G4 dendrimers (Fu et al. 2006).

Optical imaging has numerous advantages for tracking and detecting disease. For optical imaging, organic structure-based fluorescent molecules, fluorescein isothiocyanate (FITC), GFP, Cy3-fluorophores, Alexa Fluor 594, and so forth can be conjugated or load with different generations of dendrimer molecules that are utilized for cell uptake studies (Koyama et al. 2007; Waite and Roth 2009). Radioactive materials can also be conjugated with dendrimers for diagnosis and treatment. The conjugated radioactive dendrimer complex can be irradiated with gamma rays and imaged with single photon emission tomography (SPECT) and positron emission tomography (PET) (Xing et al. 2018; Zhao et al. 2017). The dendrimers can be encapsulated with metal-based nanoparticles that can be visualized inside the tissue

through transmission electron microscopy (TEM). High-density gold (Au) and silver (Ag) nanoparticles are easily visualized by TEM to locate dendrimer molecules inside the cells (Vasile et al. 2014; Kéki et al. 2000).

8.10 Conclusions

Dendrimers are demonstrated to provide an excellent platform for drug and gene delivery for cancer therapy. The unique and highly defined structure, characterized by the presence of many functional groups, allows dendrimers to incorporate or conjugate multiple agents, including chemotherapeutics, nucleic acid therapeutics, image contrast agents, and targeting ligands. Despite the promising use of dendrimers as nanoparticle drug delivery systems, the use of dendrimers in the clinic has not been successful. This is because cationic PAMAM dendrimers are more toxic than their anionic counterparts. Larger dendrimers are more toxic than smaller dendrimers of similar surface functionality. However, techniques such as PEG modification have been employed in masking cationic residues with neutral groups that improve the tolerability of PAMAM dendrimers and their uptake by the cells. With further understanding of dendrimers' molecular characteristics, interaction with biological membranes, toxicity profile, and improved synthesis, it is hoped that cancer therapy applications may be realized in the near future.

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Chapter 9
Nanoparticle Design to Improve Transport Across the Intestinal Barrier

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Abstract Overcoming the intestinal epithelium barrier is an important challenge for orally administered drugs, specifically for those that elicit poor water solubility and permeability, and several efforts have been developed to address this challenge. Nanosized drug delivery systems constitute one of these attempts to enhance the penetration and permeation of drugs through the intestinal epithelium; however, there remain limitations to be addressed to lead and elaborate an effective oral drug delivery system. A profound understanding of the mechanism of nanoparticle internalization pathways through gastrointestinal epithelial cells, i.e., endocytosis and paracellular transport, is required to overcome these pitfalls. Furthermore, the physical and chemical properties of nanoparticles, including size, shape, charge, surface composition, and particle deformation and degradation, are relevant factors that influence the internalization process and also in the toxicity profile in the organism. In addition, multiple developed and reported strategies for nanoparticles to target the intestinal epithelial cells, to adhere and penetrate the mucus gel barrier, and to perform a differentiated response according to a specific stimulus (i.e., bioresponsive effect) have been described in the relevant literature. Therefore, this chapter will provide a comprehensive depiction of key nanoparticle aspects to help to formulate a rational and effective design to overcome the intestinal epithelium barrier.

Keywords Nanoparticles · Intestinal epithelium · Drug delivery system · Intestinal barrier · Intestinal epithelium permeation · Bioresponsive nanoparticles · Nanotoxicology

9.1 Introduction

Epithelia are organized cellular structures served as natural barriers, maintaining and separating the internal environment of the body from external surfaces. An example of epithelial barriers is the epithelium covering the gastrointestinal tract, which is composed of different types of cells, so-called intestinal epithelial cells

(IECs), and closely packed to each other, forming an asymmetric cellular layer characterized with a selective permeability (Laurent et al. 2017). This ability to restrict the passage of different molecules is a great challenge for orally administrated drugs, especially those with poorly water-soluble and poorly permeable properties. As the oral route of administration is the most popular and comfortable and results in better compliance by patients, several efforts have undertaken to overcome this problem. Furthermore, chemical, physical, and biological interactions between the drug and cellular components of the epithelium are the main determinant of whether or not and to what extent the drug may reach into the blood circulation and therefore, perform the therapeutic effect (Berardi and Bisharat 2014). As such, nanotechnology has emerged as a new tool for the design and elaboration of nanosized drug delivery systems. Using different types of these nanosized systems, an effective and enhanced permeation of transported drugs can be observed (Diab et al. 2017; El-Say and El-Sawy 2017; He et al. 2018). Moreover, active targeting strategies and the development of mucoadhesive and mucopenetrating nanomaterials comprise a whole new platform for engineering an optimal drug delivery system.

The goal of this chapter is to provide an overview of several aspects that must be considered for the design and development of an orally administered nanosized drug delivery system. The first part of the chapter will focus on the main pathways of nanoparticles to penetrate and permeate the epithelial barrier. Then, the second section will detail several parameters related to the physical and chemical properties of the nanoparticle and how they can impact on its internalization process through the epithelial barrier. The third and core part of this chapter will describe several strategies to overcome the epithelial barrier using targeting ligands, mucoadhesive and mucopenetrating agents, and novel bioresponsive delivery systems. Finally, the fourth section will be dedicated to the toxicity related to nanoparticles and the impact in the organism. The overall aim of this chapter is to describe the current tools that can be employed for enhancing the permeation through the intestinal epithelial barrier using nanosized drug delivery systems.

9.2 Mechanisms of Nanoparticle Barrier Penetration and Permeation

The transport of different kinds of molecules, including nanoparticles, through different compartments of the body, leads to factors associated with crossing biological membranes. These membranes act as barriers that show selective permeability, allowing them to control the transport of molecules and cell interaction with the environment and therefore, maintaining the cell composition equilibrium and integrity. There are several mechanisms described to allow molecule transport, depending on the molecular size and concentration, or electrochemical gradient. One of these mechanisms is endocytosis and consists of the process by which cells internalize selected extracellular molecules, including lipids, proteins, nanosized

biomimetic particles, viruses, and even microorganisms, by endocytic vesicles called endosomes (Decuzzi and Ferrari 2008). Endocytosis is an active saturable internalization mechanism, i.e., a concentration, time, and energy-dependent process (Harush-Frenkel et al. 2008), and plays an important role in multiple biological processes such as mitosis, antigen presentation, cell migration, intracellular signaling cascades pathways, and nanoparticle interaction with barriers (Doherty and McMahon 2009). Among the endocytic pathways, these are classified into transcellular and intracellular mechanisms (Fig. 9.1).

9.2.1 Transcellular Pathway

The transcellular pathway is the major endocytic route for nanoparticle internalization with a very large surface area for this mechanism (Cai et al. 2010). Usually, this pathway involves the uptake of particles at the apical membrane of polarized cells, followed by release into the basolateral compartment, i.e., an endocytosis/exocytosis dual pathway, so-called transcytosis (Woitiski et al. 2008). Within the endocytic process of transcytosis, there are several mechanisms relevant to nanoparticle design:

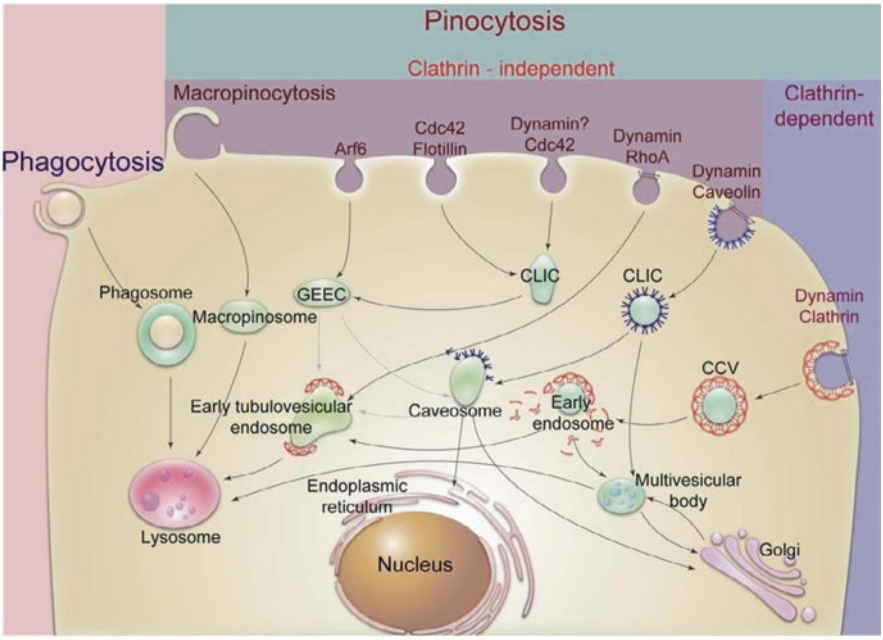

Fig. 9.1 Endocytic mechanisms of nanoparticle penetration, including transcellular and intracellular pathways. *CCV* clathrin-coated vesicle, *CLIC* clathrin-independent tubulovesicular carrier, *GEEC* glycophosphatidyl inositol anchored protein-enriched early endosomal compartment. (Reproduced with permission from Sahay et al. (2010a))

Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis (CME) is the most classical route of cellular receptor-mediated endocytic entry, which is present in all mammalian cells (Kou et al. 2013; Sahay et al. 2010a). This route fulfills important physiological functions, including nutrient uptake and intracellular communication. The CME typically occurs in a membrane region enriched with a cytosolic three-legged structure protein, clathrin 1. After ligand binding to the respective receptor, cytoplasmic motifs of the receptor engage adaptor proteins, such as AP2 and AP180, which interact with clathrin, leads to the formation of a clathrin-coated basket-like structure (Hillaireau and Couvreur 2009; Kanaseki and Kadota 1969; Rajendran et al. 2010; Sahay et al. 2010a). As clathrin structure formation continues, the pit becomes deeply invaginated into the cytoplasm until fission and formation of 100–150 nm-sized clathrin-coated vesicles, mediated by a small GTPase protein, dynamin (Pucadyil and Schmid 2009). Once these vesicles are formed and internalized, the uncoating process is developed to recycling clathrin proteins (Conner and Schmid 2003). After this process, uncoated vesicles are delivered to acidic early endosomes, after which receptors and ligands are dissociated and recycled (Hillaireau and Couvreur 2009). When early endosomes mature into late endosomes, reaching acidic conditions (pH 5), these vesicles fuse with lysosomes, and the content is exposed to enzymatic degradation (Bareford and Swaan 2007; Mukherjee et al. 1997). However, there is evidence that not all late endosomes will fuse with lysosomes. Furthermore, they can be excluded from the pathway and transported to the Golgi complex or even to the opposite membrane and be exocytosed (Matter and Mellman 1994; Rappoport 2008).

Caveolae-Mediated Endocytosis

Caveolae-mediated endocytosis (CvME) is an alternative pathway for particle internalization and is widely present in muscle, endothelial cells, fibroblasts, and adipocytes and absent in neurons and leukocytes (Doherty and McMahon 2009). This is a clathrin-independent, but dynamin-dependent endocytosis, and is mediated by caveolin, a dimeric protein (Doherty and McMahon 2009; Hillaireau and Couvreur 2009). Caveolae are characteristic flask-shaped membrane invaginations, eliciting an average size between 50 nm and 80 nm and are enriched with lipid rafts, constituted by cholesterol and sphingolipids (Hillaireau and Couvreur 2009; Sahay et al. 2010a). CvME is a highly regulated process, involving a complex signaling pathway, which may be driven by the cargo content itself (Bareford and Swaan 2007; Conner and Schmid 2003). When particles are attached to the cell surface via receptor-ligand interactions, they move along the plasma membrane to caveolae invaginations (Bareford and Swaan 2007). Once there, membrane curvature is induced by cavin protein, and fission of these caveolae structures generates cytosolic caveolae vesicles, in a dynamin-dependent process (Hillaireau and Couvreur 2009; Nabi 2009). These vesicles fuse with caveosomes or neutral multivesicular

bodies by vesicle-associated membrane protein 2 and synaptosome-associated protein (Schnitzer et al. 1995). This pathway is slower than CME, but can avoid the lysosomal degradation and therefore, could be an advantage for the delivery of highly enzymatic-sensitive drugs or delivery systems (Hillaireau and Couvreur 2009; Sahay et al. 2010a).

Nonspecific Adsorptive Pinocytosis

Nonspecific adsorptive pinocytosis is also referred to as fluid-phase endocytosis. Although this pathway is a variant of CME, there is a difference: nonspecific adsorptive pinocytosis is a receptor-independent mechanism, i.e., substances avoid direct attachment to cell membrane constituents. Nonspecific charges and hydrophobic interactions are the main mechanisms of cell adsorption, and the internalization process includes the entry of extracellular fluid and contents, carried out by clathrin-coated vesicles (Bareford and Swaan 2007; Hillaireau and Couvreur 2009). This adsorption process has a slower internalization rate compared to the classical receptor-dependent CME (Strømhaug et al. 1997).

Clathrin- and Caveolae-Independent Endocytosis

Both CME and CvME are pathways resulting in protein-coated vesicles and requiring the presence of dynamin. However, it is known that there are several mechanisms of endocytosis leading to the formation of noncoated vesicles and therefore, dynamin is not required. Among these pathways, CLIC/GEEC, Arf-6, and flotillin-associated endocytosis are the most described.

CLIC/GEEC Pathway

Clathrin-independent tubulovesicular carrier/glycophosphatidyl inositol anchored (GPI-AP)-enriched early endosomal compartments protein (CLIC/GEEC) pathway is a high-capacity route of internalization, specifically for lipid-anchored proteins (e.g., GPI-AP) and transmembrane proteins (e.g., CD44 and dysferlin) (Howes et al. 2010; Mayor et al. 2014; Rajendran et al. 2010). The structure of endocytic vesicles formed by this pathway is termed GEEC and results from the fusion of primary uncoated CLICs, derived from the cell surface (Kirkham et al. 2005). GEEC proteins appear to be enriched by GPI-APs, but the mechanism for this enrichment is not completely clear; however, a cholesterol and sphingolipid-dependent sorting mechanism is proposed, due to alterations on the level of those lipids that affects the vesicle formation in this pathway (Chadda et al. 2007; Sharma

et al. 2004). CLIC/GEEC endocytosis is initiated by the recruitment of Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1) at the cell surface and activates ADP-ribosylation factor 1 (Arf1) protein. This activation leads to maintenance in the cyclic state of cell division control protein 42 (Cdc42), essential for the downstream actin polymerization process (Mayor et al. 2014). Although CLIC formation is dynamin-independent, this protein is localized to GEECs post-internalization and plays a role in vesicle recycling (Kirkham et al. 2005).

Arf6-Associated Pathway

ADP-ribosylation factor 6 (Arf6) is a protein located at the cell surface and is involved in clathrin- and dynamin-independent, but cholesterol-dependent endocytosis (Mayor et al. 2014). After the formation of endosomes, they fuse with Rab-5-positive vesicles, which can sort for recycling or degradation. The activation of Arf6 is required for the recycling pathway, but not for endocytosis. However, inactivation of this protein is important after internalization for the sorting of endosomal cargo (Naslavsky et al. 2003). Among substances using this pathway mostly are endogenous cell surface proteins (e.g., CD44 and CD59), major histocompatibility complex class I, glucose transporter GLUT1, and carboxypeptidase E (Doherty and McMahon 2009; Mayor et al. 2014).

Flotillin-Dependent Pathway

Flotillin 1 and 2 proteins play a key role in this mechanism of endocytosis, involving both dynamin-dependent and dynamin-independent internalization (Sandvig et al. 2011). For example, basolateral internalization of GPI-APs is carried out by flotillin 2 and is a dynamin-dependent process (Aït-Slimane et al. 2009). On the other hand, flotillin 1-dependent and dynamin-independent endocytosis are relevant for the internalization of cholera toxin B (Glebov et al. 2006). Other cargoes that exploit this pathway are Shiga toxin, CD59, and proteoglycans (Doherty and McMahon 2009; Pust et al. 2010).

9.2.2 Intracellular Pathway

Intracellular pathways are mechanisms of internalization involving large volumes of the membrane, and the final destination is the lysosome and therefore, this route is a degrading internalization pathway. This classification includes phagocytosis and macropinocytosis.

Phagocytosis

Phagocytosis is defined as the recognition and ingestion process of particles larger than 0.5 μm into membrane-bound vesicles, known as phagosomes (Freeman and Grinstein 2014). This process is a clathrin-independent and actin-dependent pathway and allows the internalization of particles, pathogens, and apoptotic cells (Champion and Mitragotri 2006; Freeman and Grinstein 2014; Murugan et al. 2015). This type of internalization pathway is widely described in phagocytes, including macrophages, monocytes, and dendritic cells and plays a key role during the immune response (Freeman and Grinstein 2014). However, there is evidence that other types of cells also exert phagocytic activity, such as fibroblasts and epithelial and endothelial cells, but to a much lower extent (Rabinovitch 1995). The basic mechanism of phagocytosis includes recognition by opsonization in circulation, adhesion of the opsonized particle to the cell, and subsequent internalization. Opsonization is a process consisting in tagging foreign particles with circulating blood proteins, opsonins (e.g., immunoglobulins G and M, complement system protein fragments, laminin, fibronectin, and C-reactive protein), making them visible to macrophages (Hillaireau and Couvreur 2009; Owens III and Peppas 2006; Vonarbourg et al. 2006). After this process, opsonized particles are recognized by circulating macrophages and attached by specific receptor-mediated interactions, where Fc and complement receptors are the major and best characterized, recognizing immunoglobulin-opsonized and complement proteins-opsonized particles, respectively (Aderem and Underhill 1999; Groves et al. 2008). This receptor-ligand interaction triggers a signaling cascade mediated by GTPase Rho proteins, leading to actin assembly and the formation of cell surface extensions, so-called pseudopodia, which enclose and engulf the opsonized particles (Caron and Hall 1998). Furthermore, other receptors have been reported to be involved in the phagocytosis pathway, such as mannose/fructose scavenger and CD44 receptors (Aderem and Underhill 1999; Vachon et al. 2006, p. 44). The resulting phagosome will carry throughout the cytoplasm when actin protein is depolymerized from the vesicle and allows it to fuse with early endosomes and ultimately lysosomes, to a degradative pathway (Hillaireau and Couvreur 2009).

Macropinocytosis

Macropinocytosis is a clathrin-independent and cholesterol-dependent endocytic route and is related to large-scale internalization process, involving the formation of membrane protrusions (Doherty and McMahon 2009; Lim and Gleeson 2011). Extracellular components trapped between these protrusions are internalized in vesicles, known as macropinosomes (Doherty and McMahon 2009; Swanson 2008). These macropinosomes have no particular coating structure, and sizes are ranged between 0.2 and 10 μm and therefore, provide a pathway for nonselective internalization of a large quantity of solute (Lim and Gleeson 2011; Sahay et al. 2010a). As phagocytosis, macropinocytosis is an actin-dependent pathway, i.e., the protein

polymerization and reorganization is essential for the formation of membrane protrusions (Hillaireau and Couvreur 2009). Once macropinosomes are formed, these vesicles undergo a maturation process leading to two possible results: (1) fusion with lysosomes and degradation by lysosomal enzymes or (2) fusion with the plasma membrane and recycling the content to the cell exterior (Lim and Gleeson 2011).

9.2.3 Paracellular Pathway

Another transport mechanism to bypass epithelial barriers is the paracellular route, consisting of a non-endocytic passive transport of substances between adjacent cells (Morris and Bridget 2013, p. 5; Murugan et al. 2015). This pathway is limited to the very small surface area of intercellular spaces and tight junctions between cells and is specific for the transport of small hydrophilic molecules (Murugan et al. 2015; des Rieux et al. 2006). A pore formed by the space between adjacent cells has a variety of diameters of up to 15 Å and occupies less than 1% of the mucosal surface, compared to the transcellular pathway (Cai et al. 2010; Hayashi et al. 1997). The most relevant intercellular structures for substance transport are tight junctions and are found in most apical regions of the intercellular connections (Laksitorini et al. 2014). These structures are the rate-limiting factor of the paracellular permeation process (Ménard et al. 2010). Tight junctions are composed by a multiprotein complex of occludins, claudins (claudin-3, claudin-5, and claudin-12), junctional adhesion molecules (JAM-A, JAM-B, and JAM-C), and tricellulin and are associated with apical actomyosin rings, leading to the formation of an organized structure (Laksitorini et al. 2014; Ménard et al. 2010). These proteins have negatively charged amino acids in the composition, and thus, paracellular transport is more likely developed by small positive-charged NPs, due to electrostatic interactions (Woitiski et al. 2008). Under the steady-state condition, tight junctions allow the diffusion of cations and inert molecules of small size with a molecular weight of 600 Da or less (Ménard et al. 2010).

All transcellular, intracellular, and paracellular pathways of internalization are summarized in Table 9.1. These pathways occur within a wide range of cells, and understanding these processes can provide an approach to design drug delivery systems that exploit these types of internalization pathways for crossing epithelial barriers, especially those that avoid lysosomal fusion to prevent drug degradation.

9.2.4 Homeostasis of Intestinal Barrier

An important role of biological membranes is to provide protection serving as a barrier for exogenous and potential harming particles; however, this barrier function, especially in the intestinal epithelium, can be compromised in several types of diseases, including inflammatory and autoimmune illnesses, leading to a disrupted

Table 9.1 Summary of types of barrier penetration pathways found in transcellular, intracellular, and paracellular transport mechanisms

Type	Pathway	Mechanism	Observations
Transcellular	Clathrin-mediated endocytosis	Formation of invaginated clathrin-coated vesicles, in a dynamin-dependent process. This mechanism is mediated by ligand-receptor recognition	The main final destination of the cargo is the lysosome for degradation process; however, cargo can escape from lysosomes and be exocytosed
	Caveolae-mediated endocytosis	Formation of vesicles in a caveolin and dynamin-dependent process. This mechanism is mediated by ligand-receptor recognition	Cargo using this pathway has a slower rate of internalization in comparison with clathrin-mediated endocytosis, and it is not related to the lysosomal degradation pathway
	Nonspecific adsorptive pinocytosis	Variation of clathrin-mediated endocytosis that does not require ligand-receptor recognition	Ligand adsorption to the cell surface is required
	Clathrin- and caveolae-independent endocytosis	Some proteins and lipids are required for this type of endocytosis and are not dependent on dynamin	CLIC/GEEC, Arf6, and flotillin-dependent pathways are part of this type of endocytosis
Intracellular	Phagocytosis	Formation of phagosomes implies opsonization of circulating particles, adhesion to cell surface and internalization, and is an actin-dependent process	Solid cargoes include particles, pathogens, and cells
	Macropinocytosis	Formation of membrane protrusions (macropinosomes) incorporating cholesterol, and it is actin-dependent process	Cargoes are in a liquid state and may be destined to lysosomal degradation or membrane recycling
Paracellular	Paracellular	Formation of pores in apical intercellular connections, such as tight junctions	Transport of small positively charged particles

barrier and therefore, higher permeability (Martini et al. 2017; Turner 2006). To restore this proper function, several processes are activated, including an enhanced proliferation of epithelial cells, immune response, and regulation of gut microbes. The intestinal epithelium is composed of IECs and elicited one of the most rapid renewal processes in the human body (Roostaee et al. 2016). This tissue is renewed every 4–5 days and approximately 10^9 new cells are produced in each cycle. These new cells are derived from multipotent stem cells organized near the bottom of intestinal crypts. Several signals can induce proliferation and differentiation in several types of IECs. As these stem cells begin to differentiate, a migration process

from the base to the top of intestinal crypts occurs. Furthermore, by the time that these cells leave the crypt, they are fully differentiated in IECs and continue the migration up to the top of intestinal villi (De Mey and Freund 2013; van der Flier and Clevers 2009). Another important homeostatic process is related to the immune response in the intestinal lumen and is associated with the secretion of immunoglobulin A and antimicrobial peptides. Both molecules are involved in the protection against bacterial invasion of the mucus layer and secreted in intestinal dysbiosis-related diseases, reinforcing the integrity of the intestinal barrier and cushion cytokines-related proinflammatory responses (Okumura and Takeda 2017; Wells et al. 2017). In addition, gut microbiota and metabolites contribute to the mucosal barrier function of IECs, influencing tissue and immune development, providing metabolic functions and colonization resistance against pathogens (Okumura and Takeda 2018; Wells et al. 2017). Therefore, a well understanding of the intestinal barrier homeostasis, i.e., epithelium cells renewal cycles, immune response, and microbiota and metabolite interactions, may provide advantages in the design and proper formulation of drug delivery systems.

9.3 Nanoparticle Properties Governing Barrier Transport

An important issue that nanoparticles, as drug delivery systems, should achieve is to reach the absorptive epithelium surface, promote interactions with cells, and therefore, cross this barrier and reach the systemic circulation. These critical nanoparticle–cell interactions are influenced by several physical and chemical parameters of nanoparticles for drug delivery, including particle size, shape, surface charge, surface chemistry, and particle deformability and degradability (Fig. 9.2).

9.3.1 Nanoparticle Size

The particle size is an important key parameter that represents the three-dimensional in a one-dimensional singular scale value. Decreasing in particle size has been related to distinct properties about the bulk material that are responsible for several improvements in biological effects of nanoparticles used as drug delivery systems, including cell internalization rate, circulation half-lives, extravasation, or penetration through vasculature and mononuclear phagocyte system uptake (Blanco et al. 2015; Yokoyama et al. 2008).

As mentioned before, the cell internalization process is influenced by particle size. In this context, it has been described that an enhanced and easier internalization by epithelial cells is achieved using small-sized particles and therefore, can extravasate into circulation to reach diseased tissues, including inflamed endothelial cells and tumors (Sahay et al. 2010a; Singh and Lillard Jr. 2009). Nanoparticles with size ranging from 50 nm to 200 nm have been shown to have greater cell

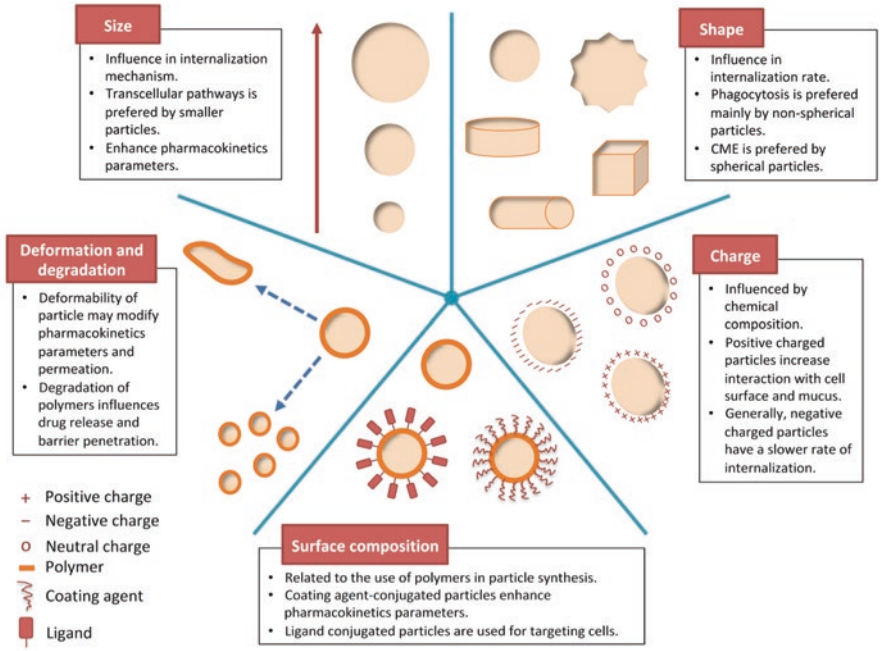

Fig. 9.2 Nanoparticle properties governing barrier transport and permeation, including particle size, shape, charge, surface composition, and deformability and degradability. *CME* clathrin-mediated endocytosis, *NP* nanoparticle, *PEG* poly(ethylene glycol), *PVP* poly(vinyl pyrrolidone)

internalization rate compared with larger particles using both in vitro and in vivo models of intestinal epithelial cells (Banerjee et al. 2016b; Florence et al. 1995; Li et al. 2015). While larger nanoparticles are internalized using intracellular-dependent pathway, specifically phagocytosis and macropinocytosis, small particles are prone to be internalized by transcellular-dependent mechanism, either by CVE or CvME (Koval et al. 1998; Li et al. 2015; des Rieux et al. 2006).

9.3.2 Nanoparticle Shape

The shape of a nanoparticle is a parameter related to size and therefore, it must be considered at the design of delivery systems. As the size, particle shape can also influence several phenomena in biological tissues and organs. To date, the most described shape is spherical, but in the past decades to discoid, rod-like, filamentous, cylindrical, and barrel-shaped NPs are been obtained (Champion et al. 2007; Kinnear et al. 2017). There is evidence that nonspherical-shaped nanoparticles elicited an enhanced cell internalization rate compared with spherical ones (Banerjee et al. 2016b; Chithrani et al. 2006; Hao et al. 2012; Zheng et al. 2017). For example,

Hao et al. have developed different types of poly(ethylene glycol) (PEG)-conjugated fluorescent mesoporous silica nanoparticles, including spherical, short rod, and long rod-shaped, and demonstrated that long rod-shaped particles achieved the highest cell internalization rate compared with the other two shapes (Hao et al. 2012). The difference observed can be explained by the larger contact area interaction of rod-shaped in contrast with spherical particles. Furthermore, the shape also influences the internalization pathway. The same study of Hao et al. has demonstrated that spherical particles are internalized via CME, long rod-shaped prefer CvME, and short rod-shaped can be internalized indistinctly by both mechanisms (Hao et al. 2012). Therefore, the shape of the particle is an important parameter that influences the rate and mechanism of cell internalization.

9.3.3 Surface Charge

The surface charge or zeta potential is a parameter related to the charge of a structure and represents the electrostatic potential mean in the particle surface (Hunter 1981; Woitiski et al. 2008). Chemical nature of the nanoparticle surface, the stabilizing agent used in the elaboration process, and pH of the medium are major factors that influence the net surface charge (Mora-Huertas et al. 2010). There is consensus that positively charged particles elicited a higher interaction with negatively charged cell membranes or other components surrounding the cells, e.g., negatively charged mucin proteins forming the mucus layer (Gratton et al. 2008; Norris and Sinko 1997). Chitosan nanoparticles may interact with negatively charged groups of the mucosa. This is a biocompatible, biodegradable, hydrophilic, and positively charged polymer and can interact with negatively charged groups of the cell membrane and mucus layer and therefore, be used to design nanoparticles for crossing biological barriers (Behrens et al. 2002; Cai et al. 2010). Therefore, the interaction between positively charged particles and the negatively charged constituents of the cell membrane and mucus layer enhances the cell internalization rate of these nanoparticles. This was confirmed by Ha et al. and demonstrated a positive correlation between an increase in the zeta potential of β-lactoglobulin nanoparticles and the cell internalization rate using an experimental in vivo Caco-2 cell model (Ha et al. 2015). Rather than advantages of positively charged nanoparticles, there are negatively charged nanosystems reported to be more prone to be internalized, including cadmium selenide core and zinc sulfide shell quantum dots, gold, and polystyrene nanoparticles (Walczak et al. 2015a, b; Zhang and Monteiro-Riviere 2009). Furthermore, Harush-Frenkel et al. have shown that positively charged poly(lactic acid) (PLA)-PEG nanoparticles are internalized by CME, and when this pathway is saturated, the uptake is driven by macropinocytosis. Negatively charged PLA-PEG particles are internalized to a lower extent by CME and clathrin-dynamin independent pathways, and similarly to positive ones, when these mechanisms are saturated, these particles are internalized via macropinocytosis (Harush-Frenkel et al. 2008). Other studies indicate that negatively charged liposomes, micelles, and

quantum dots are possibly internalized via CvME (Sahay et al. 2010b; Zhang and Monteiro-Riviere 2009). Furthermore, Lin et al. have demonstrated that negatively charged gold nanoparticles cross the epithelial Caco-2 cell monolayer through tight junctions via the paracellular route, while positive and neutral gold particles are internalized by endocytosis (Lin et al. 2012).

9.3.4 Surface Properties of Nanoparticles

The surface properties are related mainly by the chemical composition of nanoparticles and associated with particle cell internalization. Indeed, a balanced composition of hydrophobic and hydrophilic moieties at the particle surface leads to an optimal cell internalization process (Cai et al. 2010). In this parameter, the polymer chosen to synthesize drug delivery systems must be biocompatible and biodegradable. Among these, poly(lactic-co-glycolic acid) (PLGA) and chitosan have been used for this purpose. PLGA nanoparticles have been applied for vaccines administration (Slütter et al. 2010; Tian and Yu 2011) and targeting to cancer cells (Amin et al. 2016; Aravind et al. 2012) and have been reported that are internalized via fluid-phase pinocytosis and CME (Danhier et al. 2012). On the other hand, chitosan is also used as mucoadhesive particles and internalized mainly by adsorptive-mediated endocytosis and CME in respiratory epithelial A549 cells (Huang et al. 2002). However, there is evidence that chitosan nanoparticles can also be internalized by CME using an in vitro Caco-2 cells model (Ma and Lim 2003). Furthermore, another important property using chitosan is the ability to temporarily open tight junctions to enhance paracellular transport (Vllasaliu et al. 2010; Zhang et al. 2014a). Covering the particle surface with hydrophilic polymers, including PEG and poly(vinyl pyrrolidone), has been reported to decrease the opsonization process and prevent the uptake of nanoparticles by mononuclear phagocyte system tissues, mainly due to steric repulsive forces present at the surface of these nanoparticles against circulating proteins. This phenomenon leads to an enhanced circulation time of nanoparticles in the so-called stealth effect (Gref et al. 2000; Ogawara et al. 2004; Owens III and Peppas 2006; Perrault et al. 2009; Rabanel et al. 2012; Torchilin et al. 1994).

9.3.5 Deformability and Degradability of Nanoparticles

Another feature that is relevant in nanoparticle design to improve the ability to cross a biological barrier is related to the deformability and degradability of nanoparticles. The deformability is related to the elasticity and ability of a particle to adopt different forms and therefore, may contribute to the modulation and performance of several pharmacokinetic parameters. For example, rigid particles with a greater diameter than the cutoff limit of fenestrations of splenic interendothelial slits are

prone to be cleared by this organ (Blanco et al. 2015). Cui et al. have developed PEG hydrogel particles with tunable elasticity and demonstrated that super soft hydrogels, i.e., particles with high elasticity and thus, an increase ability to deform, are more susceptible to pass through the microchannels in a microfluidic blood capillary model (Cui et al. 2014). Recent studies have also confirmed that deformability is directly relevant for tumoral cells uptake and to optimally penetrate intestinal mucosal and tumor matrix of nanolipogels and PLGA-lipid nanoparticles (Guo et al. 2018; Yu et al. 2018). On another key aspect of nanoparticles, degradability has a major impact on the release of the encapsulated drug and also influences the stability of the delivery system. In this matter, biodegradability is a critical parameter to design a nanoparticle, i.e., using constituents that are proved of high biocompatibility and the degradation products are non-toxic and not cause adverse effects (Blanco et al. 2015). Furthermore, the biodegradability of nanoparticle constituents is related to barrier penetration. Tang et al. have developed biodegradable poly(sebacic acid) and PEG nanoparticles and demonstrated an enhanced permeation through human cervicovaginal mucus in comparison with polystyrene nanoparticles, due to effect partitioning of PEG units and therefore, may constitute a valid platform for controlling drug release (Tang et al. 2009). Thus, nanoparticle-related parameters, including particle size, shape, charge, surface properties, deformability, and degradability, are important to be considered in nanoparticle development as a drug delivery system and influence their ability and performance of crossing biological barriers, by promoting interactions between nanoparticle and cell surface.

9.4 Strategies to Improve Nanoparticle Transport Across the Intestinal Barrier

The safety and noninvasiveness made the oral route an ideal route of administration for drugs and delivery systems. Although these advantages, the administration of poorly water-soluble and poorly permeable drugs are always been difficult and challenging, due to the poor bioavailability, and consequently the pharmacological effect. For this reason, nanoparticles as drug delivery systems have emerged as a solution to this disjunctive. Nonetheless, crossing the intestinal epithelium and therefore, reaching systemic circulation remains a challenging feature that must be addressed for an efficient delivery and effect of the drug.

Anatomically, the larger absorption surface for nutrients, drugs, and other substances in the gut is present in the small intestine and provides efficient barrier for foreign particles, including toxins, peptides, and microorganisms (Lundquist and Artursson 2016). This barrier effect is due to several processes, including pH environment, the presence of hydrolytic enzymes, the mucus gel layer, and the organization of the epithelial cells (Lee and Yamamoto 1989). The epithelium of the small intestine is composed of several types of cells, called IECs, and these cells are prone

to internalize and transport substances from intestinal lumen to systemic circulation. Therefore, several strategies have been proposed to enhance absorption and internalization processes for nanoparticles (Fig. 9.3).

9.4.1 *Enterocyte-Mediated Transport*

Enterocytes are the most numerous cells among IECs (Peterson and Artis 2014). The apical surface has multiple projections known as microvilli and formed a highly specialized structure for efficient digestion, substance absorption and transport, and protecting barrier, called brush border (Delacour et al. 2016). The internalization process of nanoparticles can be achieved by exploiting the existing knowledge about receptors used for the absorption of molecules, including antibodies, transferrin, vitamins, and monocarboxylate substances.

A very known antibody receptor is the neonatal Fc receptor (FcRn), which binds to immunoglobulin G and also albumin. A very unique characteristic of this receptor involves a pH-dependent activity, i.e., this receptor can bind ligands in the apical side at a mildly acidic environment (pH < 6.5) and then be internalized and cross the whole cell by a transcytosis mechanism and release the ligands in the basolateral side at physiological pH (7.4) (Pridgen et al. 2013; Pyzik et al. 2015). Pridgen et al. have developed immunoglobulin G-conjugated PLA-PEG nanoparticles and demonstrated an augmented transepithelial transport using an in vitro Caco-2 cell model, compared with nonconjugated particles. The administration of human

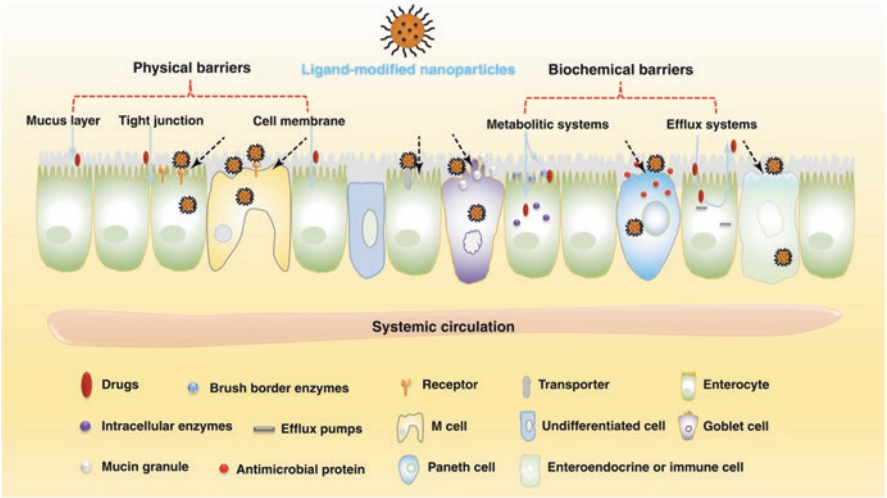

Fig. 9.3 Illustration of the absorptive small intestinal epithelium and intestinal epithelial cells for potential active targeting of nanoparticles to improve transport across the intestinal barrier. (Reproduced with permission from Zhang and Wu (2014))

immunoglobulin G solution also impaired the internalization rate of these conjugated nanoparticles, due to the higher affinity of free human immunoglobulin G and FcRn receptor, demonstrating that these immunoglobulin G-conjugated nanoparticles can bind and be internalized by a FcRn-dependent pathway (Pridgen et al. 2013). Recently, Shi et al. have developed PEG-PLGA-nanoparticles modified with Fc and loaded with exenatide, a hypoglycemic drug, and demonstrated a faster and greater permeation rate in comparison with unmodified particles, using in vivo Caco-2 cells model. Furthermore, orally administered of these modified nanoparticles have elicited an extended hypoglycemic effect in mice compared with subcutaneous injection of the exenatide solution (Shi et al. 2018). However, the main disadvantage of this type of targeting involves the production and therefore, the cost of FcRn ligands that may require eukaryotic expression systems due to specific patterns of these ligands, including glycosylation or disulfide bonds (Sockolosky and Szoka 2015).

On the other hand, the transferrin receptor elicits a high affinity for transferrin, a non-heme iron-binding glycoprotein, important for dietary iron absorption (Qian et al. 2002). Some evidence demonstrates the overexpression of this receptor in central nervous systems and tumor cells (Högemann-Savellano et al. 2003; Ulbrich et al. 2009). Besides, the transferrin receptor is involved in a CME-dependent internalization process (Grant and Donaldson 2009). Insulin conjugated with transferrin to target transferrin receptor in enterocytes has elicited 5–15-folds higher internalization rate using an in vitro Caco-2 cell monolayer model and demonstrated a higher hypoglycemic effect after oral administration of streptozotocin-induced diabetic rats, compared with free insulin (Lim and Shen 2005; Shah and Shen 1996). Micelles made by transferrin receptor-specific 7 peptide-conjugated PEG-*b*-polycaprolactone and loaded with coumarin 6 have demonstrated an increased internalization rate in Caco-2 cells and higher accumulation in late endosomes and lysosomes, compared with nonconjugated micelles (Du et al. 2013). Although the enhanced permeation in enterocytes, transferrin-conjugated nanoparticles may be harmful to low transferrin receptor-expressed cells causing toxicity and adverse effects (Alexander-Bryant et al. 2013).

Vitamins can also be used as targeting ligand, and complex B is the most used. Folate (vitamin B9) is a water-soluble vitamin and is important for nucleotide synthesis as well as for the methylation process of nucleic acids, proteins, and phospholipids (Dai and Koh 2015). It has been described that the internalization process of this vitamin in enterocytes is related to two different proteins: intestinal reduced folate carrier and proton-coupled folate transporter (Visentin et al. 2014). Paclitaxel-loaded folate-PEG-PLA-conjugated PLGA nanoparticles have demonstrated a higher internalization rate of this poorly water-soluble antitumoral drug, using an in vitro Caco-2 cells model, related to the expression of folate carrier in a CvME-dependent pathway (Roger et al. 2012). Furthermore, folate-poly(ethyleneimine)-conjugated mesoporous carbon nanoparticles have elicited an enhanced internalization rate, for the same drug and using an identical Caco-2 cell culture (Wan et al. 2015). However, the main drawback of using folate as targeting ligand

is that the site of conjugation is difficult to precisely control, leading to the formation of a heterogeneous population of folate conjugates (Zhao et al. 2008).

Cyanocobalamin (vitamin B12) is another water-soluble vitamin and absorption depends on a glycoprotein known as intrinsic factor. This complex is internalized by cubilin, the specific endocytic enterocyte receptor (Kozyraki et al. 1999). Vitamin B12-conjugated Gantrez AN poly(methyl vinyl ether-co-maleic anhydride) nanoparticles linked with dimethylformamide chains elicited 3.5-fold higher tropism for distal sites of the intestine, compared with nonconjugated nanoparticles (Salman et al. 2008). Furthermore, a novel insulin-loaded calcium phosphate nanoparticles conjugated with vitamin B12-chitosan layer-by-layer coating have shown a higher internalization rate using in vivo Caco-2 cells model, mediated by cubilin-mediated endocytosis and enhanced paracellular route, by chitosan-associated opening tight junctions effect. This increased internalization rate leads to a more efficient hypoglycemic effect after oral administration of these conjugated nanoparticles, in streptozotocin-induced diabetic Wistar rats (Verma et al. 2016). Using a similar vitamin B12-amphiphilic chitosan nanoparticle, Wang et al. have shown an enhanced uptake and permeation of these particles carrying scutellarin, an active flavone, using in vitro Caco-2 cells model, as a possible treatment of type II diabetes induced-retinopathy (Wang et al. 2017). However, the main disadvantage using systems with cyanocobalamin as targeting molecule is the high accumulation rate in the liver and kidneys, due to interaction with transcobalamin II transporter and therefore, leading to high clearance of these systems (Alberto 2010).

Another water-soluble vitamin that could be used as targeting ligand is biotin (vitamin B7 or vitamin H). This micronutrient has dietary and colonic bacterial sources and is transformed to free biotin. This free biotin can be internalized by a saturable and sodium-dependent carrier-mediated process (Said 2002, 2009). Biotin-ricinoleic acid and biotin-12-hydroxystearic acid-conjugated with the antiviral drug acyclovir have elicited higher cell accumulation in MDCK-MDR1 and Caco-2 cells, indicating that both biotin (as targeting moiety) and lipid acids (as lipid raft to impart lipophilicity) act synergistically toward cell internalization process (Vadlapudi et al. 2012). Furthermore, oral administration of insulin-loaded biotin-conjugated liposomes showed a significant hypoglycemic effect after treating diabetic rats, compared with conventional liposomes (Zhang et al. 2014b). A possible limitation using biotin-conjugated nanoparticles is the strong interaction between this targeting ligand and the receptor, leading to a difficult releasing process of modified nanoparticles and therefore, a highly concentrated biotin solution must be used to reverse this ligand-receptor interaction (Jain and Cheng 2017).

Monocarboxylate substances, such as pyruvate, lactate, and ketone bodies (acetoacetate and β-hydroxybutyrate), are internalized in enterocytes by proton-linked pathway monocarboxylate transporters (MCTs), specifically by solute carriers (SLC), SLC16 family (Halestrap 2012). The MCT1 isoform is the most studied and widely expressed in the liver parenchymal cells, tubule cells of the kidney, blood-brain barrier, and IECs. Furthermore, the absorption of certain drugs, including salicylate, valproic acid, atorvastatin, nateglinide, and nicotinic acid, is dependent on MCT proteins (Halestrap and Wilson 2012) and also related with the

γ-hydroxybutyric acid transport (Lam et al. 2010). Therefore, a novel butyrate-conjugated lipid-polymer nanoparticle with PEG chains, used as drug delivery system, has demonstrated an increase in cell internalization rate using an in vitro model using E12 cells, by MCT-dependent mechanism, compared with nonconjugated particles (Wu et al. 2017).

Hence, several strategies for enhanced permeation using receptors expressing in enterocyte apical cell membranes including immunoglobulins, transferrin, vitamins, and monocarboxylate compounds as targeting ligands. Although these developed strategies and the presence of microvilli in the apical surface of enterocytes, targeting to these cells may include challenges, such as high expression of hydrolytic enzymes and dense mucus layer on top of the apical membrane that influence the nanoparticle permeation process and is not addressed with the use of in vitro cell models.

9.4.2 Microfold Cell-Mediated Transport

Microfold cells (M-cells) are the second most abundant IECs and constitute the follicle-associated epithelium in Peyer's patches. The main difference between enterocytes and M-cells is related to the morphological structures. The apical surface of the latter is lack of an organized brush border, with short irregular microvilli and no extensive glycocalyx, as shown in Fig. 9.3 (Corr et al. 2008). At the basolateral surface, M-cells elicit an invagination leading to a few microns distance with the apical surface (Neutra et al. 1996). These distinctive surfaces are demonstrated to be important for the active transport of food antigens and microorganisms, mainly by endocytosis or phagocytosis-dependent mechanisms (Clark et al. 2001; Schulz and Pabst 2013). Therefore, several strategies have been developed to target these cells.

For specific M-cell targeting, lectins can be used as targeting ligands. Lectins are proteins or glycoproteins that have been reported to be useful for selective targeting of certain monosaccharides in glycans and glycolipids presenting on the M-cell surface (Becker and Lowe 2003). A specific lectin is *Ulex europaeus* agglutinin 1 (UEA-1) obtained from gorse seeds and elicits high affinity and specificity for L-fucose (Brayden et al. 2005). Monophosphoryl lipid A-loaded UEA-1-conjugated PLGA lipid nanoparticles were developed for oral vaccine delivery system and demonstrated an effective internalization using both in vitro Caco-2/Raji B co-culture and ex vivo BALB/c mice models (Ma et al. 2014). However, the main disadvantage of using UEA-1 is that the L-fucose receptor is not expressed in human M-cells, and this lectin elicits a toxic effect and is prone to intestinal degradation (Brayden et al. 2005). For these reasons, nontoxic and low-degraded lectins have been tested, including tomato lectin and wheat germ agglutinin. Both lectins have an affinity to *N*-acetyl-D-glucosamine expressed in human M-cells and have been tested as lectin-modified insulin liposomes, demonstrating to cause an effective

hypoglycemic effect compared with non-coated insulin liposomes using in vivo mice model (Zhang et al. 2005).

Another strategy used for M-cell targeting is using microorganism-derived ligands. In this sense, poliovirus type-1 and the attenuated Sabin strain have been related with selective adhesion to human M-cells, mediated by CD155 receptor, and the internalization process is related to CME-dependent pathway (Iwasaki et al. 2002; Neutra et al. 1996). *Clostridium perfringens* enterotoxin is another strategy that could be used for M-cell targeted delivery systems. This toxin elicits high affinity with claudin-4, an integral membrane protein responsible for the formation of tight junctions in epithelial cells and therefore, paracellular communication (Günzel and Yu 2013). Using this toxin as targeting ligand, modified chitosan-based complexes have been evaluated as DNA vaccine model for protection against viral myocarditis induced by Coxsackievirus B3 and demonstrated that this vaccine system showed an effective immune system stimulation after oral administration in BALB/c mice, eliciting higher mucosal IgA expression and T cell proliferation, mainly interferon-γ-producing T cells, compared with nonconjugated chitosan DNA control (Ye et al. 2014). However, using microorganism-derived compounds as targeting ligands may trigger a local immune response and alter the permeation process through M-cells.

The arginine-glycine-aspartic acid (RGD) tripeptide is useful as an alternative for M-cell targeting. This tripeptide is recognized as the minimal sequence for integrin receptor recognition. Integrins are proteins that play important roles as cell adhesion receptors and several pathological processes, including cancer (Barczyk et al. 2010). Using an in vitro human M-cell model, RGD-conjugated to PEGylated PLGA nanoparticles elicited a higher internalization rate compared with the enterocytes model, demonstrating the RGD and integrin receptor interactions and an M-cell selectivity (Garinot et al. 2007). A pentapeptide–glycine–arginine–glycine–aspartic acid–serine (GRGDS)-conjugated β-glucan positively charged nanoparticles designed as oral vaccine delivery system for negatively charged PR8 antigen, an inactivated antigen of influenza A virus, have elicited a higher affinity to M-cell model compared with enterocyte cells model, due to the RGD-integrin receptor-mediated targeting. In vivo analysis for immune response in female Swiss albino rats confirmed the usefulness of this oral vaccine delivery system for immunization (Lee et al. 2017).

Thus, M-cell targeting strategies are alternatives to overcome the intestinal barrier and enhance the permeation effect of nanoparticles. Although the advantages of M-cells related to the morphology, a low number of these cells in comparison with enterocytes are the main pitfall of the development of these targeting strategies.

9.4.3 Goblet Cell-Mediated Transport

Goblet cells are specialized IECs with a high secretory function, related to the production of mucosal components, such as mucins, and several other proteins of glycocalyx (McCauley and Guasch 2015). A known strategy to target this type of

epithelial cell is using CSKSSDYQC (CSK) peptide sequence. Trimethylated chitosan chloride nanoparticles for orally insulin administration were conjugated with CSK peptide and elicited higher internalization rate through in vitro goblet-cell like HT29-MTX cell model, compared with non-modified particles, in both CME- and CvME-dependent manners (Jin et al. 2012). Novel amphiphilic dodecylamine-grafted-γ-polyglutamic acid copolymers conjugated with *N*-trimethylated chitosan-CSK peptide micelles have elicited significantly higher internalization rate in an in vitro Caco-2/HT29-MTX-E-12 co-culture cell model, compared with non-CSK-conjugated chitosan micelles. Furthermore, using this vehicle for orally insulin administration in diabetic rats, a prolonged hypoglycemic effect was observed (Zhang et al. 2015a). However, a mucus layer and the presence of mucins cover the apical face of goblet cells, as enterocytes, and may hinder the effective targeting process.

9.4.4 Dendritic Cell-Mediated Transport

Dendritic cells are not present in the intestinal epithelium, but reside below this, in the lamina propria layer. These cells have an important role in the modulation of innate and adaptive immune activities as specialized antigen-presenting cells (Coombes and Powrie 2008). Dendritic cells can only extend projections between IECs, known as dendrites, and therefore, be exposed to the intestinal lumen and could be taken advantage for absorption and internalization of drug delivery systems, mainly by the expression of surface receptors, including toll-like receptors and Fc receptors (Schulz and Pabst 2013; Zhang and Wu 2014). Three different peptide sequences, composed of 12 amino acids (FYPSYHSTPQRP, AYYKTASLAPAE, SLSLLTMPGNAS), have been proved to target dendritic cells and efficiently activate $CD4^+$ and $CD8^+$ T lymphocytes (Curiel et al. 2004). Using the first peptide as a dendritic cell-targeting molecule to deliver *Bacillus anthracis* protective antigen using *Lactobacillus acidophilus* as a vector was synthesized for vaccine strategy and showed higher protective immune response compared with non-dendritic cell-targeting vector (Mohamadzadeh et al. 2009). However, this type of targeting is exclusively developed for the immunization process.

9.4.5 Overcoming the Intestinal Mucus Gel Barrier

The intestinal epithelium at the luminal side is covered by a viscous liquid, so-called mucus barrier. This mucus is constituted by water and mucins, secreted by goblet cells, and also contains digestive intestinal enzymes and antibodies. The thickness of this mucus layer could vary according to the intestine portion, from 10 μm to 100 μm (Lundquist and Artursson 2016; Pearson et al. 2016). The contact of several small agents, including microorganisms and drug delivery systems, with IECs,

could be avoided and prevented by the presence of this mucus layer (Pelaseyed et al. 2014). Therefore, strategies have been developed to overcome this mucus barrier for efficient interaction between drug delivery systems and IECs.

The mucoadhesive effect is based on the enhanced adhesion property of a particle to the components of the mucus layer, and therefore, an increase in the residency time of a particle in the intestine is seen. This effect may be achieved using a designed positive surface charge of drug delivery systems, due to formation of electrostatic interactions with negatively charged functional groups of mucins, including sialic acid and sulfate groups (Malhaire et al. 2016). Positive charges of chitosan make this polymer an optimal component for the development of drug delivery systems with the mucoadhesive property. Indeed, trimethylated and thiolated chitosan nanoparticles have been proved for this purpose and demonstrated a significantly higher mucus adhesion capacity (Millotti et al. 2011; Yin et al. 2009). Poly(acrylic acid) is another polymer with mucoadhesive property tested. Poly(acrylic acid)-conjugated nanoparticles for oral delivery of acyclovir elicited an enhanced mucoadhesion ability using in vitro excised Sprague Dawley small intestine evaluation, and this effect is higher as increasing concentration of polymer is used (Bhosale et al. 2011). Some evidence demonstrates Eudragit® polymer systems also elicit mucoadhesive property and were successfully tested for orally insulin administration (Banerjee et al. 2016a; Zhang et al. 2015b). Mucus-penetrating particles have been developed based on the mucoadhesive property. These mucus-penetrating particles consist of nanoparticles coated with good wettability agents, promoting the formation of chemical interactions between mucus and these particles (Malhaire et al. 2016). The most used coating agent is PEG, due to the neutral uncharged and hydrophilic surface properties (Lai et al. 2009). Evidence demonstrated that PEG-coated nanoparticles enhance the adhesion and penetration of intestinal mucosa. Furthermore, low molecular weight PEG elicited a better adhesive and penetrating capabilities compared with high molecular weight polymer (Inchaurraga et al. 2015; Yoncheva et al. 2007; Zabaleta et al. 2012). An interesting topic using PEG is the surface distribution. PEG can adopt two distribution patterns on particle surface: mushroom-like distribution is associated with a low density of PEG, leading to a thin coating layer; and brush-like distribution is related to fully extended arrangement of PEG, resulting in a thicker layer (Perry et al. 2012). There is evidence that described the advantages of using the brush-like distribution for PEG-coated nanoparticles, enhancing the tissue diffusion, distribution, and several pharmacokinetic parameters (Lee and Larson 2016; Perry et al. 2012; Xu et al. 2015). Novel dissociable "mucus-inert" hydrophilic *N*-(2-hydroxypropyl)methacrylamide copolymer coating for insulin-loaded *N*-trimethylated chitosan nanoparticles has elicited a significantly higher mucus penetration, due to the dissociation of the copolymer as particles permeate through this layer, and the subsequent insulin-loaded *N*-trimethylated chitosan nanoparticles can interact with IECs and be transported by paracellular-dependent pathway, related with chitosan-induced tight junctions opening mechanism (Liu et al. 2016).

The other strategy to overcome the mucus gel layer is the mucodiffusion effect, which consists of the disruption of this mucus layer. Papain, a mucoglycoprotein

cleaver enzyme, has been used in the synthesis of mucodiffusive nanoparticles. Papain-grafted poly(acrylic acid) nanoparticles are developed as drug delivery systems and demonstrated an effective mucus penetration (Köllner et al. 2015; Müller et al. 2014). Another mucolytic enzyme used is bromelain and has been demonstrated that this enzyme elicited higher penetration efficiency than papain (Pereira de Sousa et al. 2015). *N*-acetyl-L-cysteine, another mucolytic enzyme, has been shown to reduce the cross-linking structure between mucins by breaking disulfide bonds, resulting in a decrease of mucus viscosity (Dünnhaupt et al. 2015; Liu et al. 2015). Nanomicelles made by *N*-acetyl-L-cysteine-functionalized chitosan-vitamin E succinate have been developed using this strategy for orally paclitaxel delivery and demonstrated greater mucoadhesion and mucodiffusion properties (Lian et al. 2013). Furthermore, *N*-acetyl-L-cysteine-PEG-monostearate nanostructured lipid carriers loaded with curcumin elicited higher accumulation in the duodenum of mice, compared with non-mucolytic PEG-monostearate systems, after intragastrical administration (Tian et al. 2017). Therefore, addressing the challenge of overcoming the mucus gel barrier using mucoadhesion and mucodiffusion processes may lead to effective contact and interaction between nanoparticles and the apical membrane of IECs.

9.4.6 Avoidance of Efflux Pumps

P-glycoprotein (P-gp) is a well-characterized membrane transporter and plays a key role in drug efflux at the intestinal epithelium. This protein acts as a physiological pump by extruding foreign substances out of the cells, including toxins and some drugs (Amin 2013; Srivalli and Lakshmi 2012). Although P-gp can recognize, bind, and transport several non-related drugs, almost all of the substrates are basic or uncharged and hydrophobic compounds (Lin and Yamazaki 2003, p. 59–98). To avoid this efflux pump, P-gp inhibitor-conjugated delivery systems have been described. In this sense, PEG has demonstrated that significantly inhibit the efflux and basolateral to apical transport mechanisms of several drugs, including paclitaxel, doxorubicin, and famotidine, using in vitro Caco-2 cells model (Hugger et al. 2002; Mokhtar et al. 2017).

9.4.7 Bioresponsive Delivery Systems

A novel strategy used for the efficient delivery of drugs has been proposed and is known as a bioresponsive delivery system. This type of drug delivery system is designed to be inactive in circulation and be activated by a specific endogenous stimulus at the desire tissue or cell (Lühmann and Meinel 2016). This specific endogenous stimulus is based on differences between physiological and pathological conditions, including temperature, pH value, the activity of enzymes, and

the presence of reactive oxygen species (Lühmann and Meinel 2016). These bioresponsive delivery systems possess a fragment of small peptide motif used as a linker, including the desired sequences for activation by any stimulus mentioned above (Liu et al. 2014; Weinstain et al. 2014).

One of the main bioresponsive linkers used is based on pH variation. Therefore, these pH-sensitive drug delivery systems are designed to respond selectively according to a pH value of normal or diseased tissue (Liu et al. 2014). There is evidence of these pH-sensitive delivery systems used with mesoporous silica nanoparticles. Positively charged mesoporous silica nanoparticles for sulfasalazine oral administration have demonstrated pH-differentiated responses, where higher drug release was achieved under simulated intestinal pH (7.4) compared with simulated gastric acidic conditions (1.2) (Lee et al. 2008; Popat et al. 2014). This pH-responsive effect is based on changes in the hydrodynamic diameter of nanoparticles, eliciting a higher release profile when a swollen state is reached at physiologic conditions (Chang et al. 2013). Hydroxypropyl methylcellulose phthalate-conjugated thiolated chitosan nanoparticles have also elicited pH-responsive effect used for low molecular weight heparin oral administration (Fan et al. 2016).

Another type of bioresponsive effect is to design linkers that respond to extracellular or intracellular enzymes, depending on the targeting level. In the latter case, to target an intracellular destiny, this system must be internalized using a strategy described above. A matrix metalloproteinase-sensitive linker peptide conjugated with myostatin inhibitor to solid surfaces has the potential to use for muscle-wasting disease, due to local upregulation of metalloproteinases (Braun et al. 2017). Indeed, this strategy can be used for diagnostic purposes. For example, Ritzer et al. have developed a diagnostic peptide sequence-loaded chewing gum to discriminate saliva from patients with peri-implant disease versus asymptomatic patients (Ritzer et al. 2017). PEGylated mesoporous silica nanoparticles with a shell constituted by disulfide-cross-linked poly(*N*-vinylcaprolactam-*co*-methacrylic acid) have been reported to respond to glutathione enzyme exposure, leading to reductive cleavage of disulfide bond of the cross-linker, and together with reductive pH conditions, a most rapid release of drug was achieved (Chang et al. 2013). Succinylated soy protein isolate-linked amine-functionalized mesoporous silica nanoparticles forming amide bonds have been designed to be responsive to pancreatin enzymes, capable to cleave the amide bond (Popat et al. 2014). Therefore, enzyme-triggered bioresponsive ligands can be potentially developed for enhancing the permeation process through the intestinal barrier.

The linker structure allows the bioresponsive activation of cell-penetrating peptides (CPPs), due to proteolytic cleavage for the internalization of the loaded drug into the cytoplasm of the cell (Fig. 9.4). A polyanionic counterpart, connected by a peptide sequence, neutralizes the polycationic CPP-coupled drug delivery system. This peptide sequence is cleavable by specific proteases located in tissues, and this CPP can interact with the surface of targeted cells, and then the internalization of the delivery system occurs. Masking of the positive charges exposed by the drug delivery system may contribute therefore not only to release and internalization of the drug in the presence of specific proteases in diseased tissues but also can

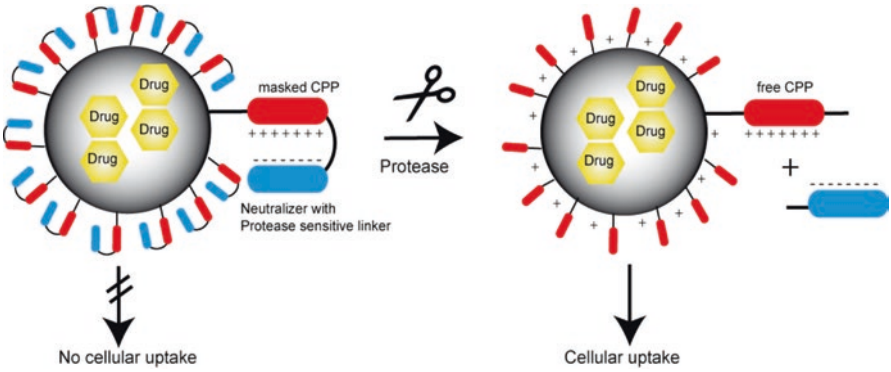

Fig. 9.4 Bioresponsive uptake of cell-penetrating peptide (CPP) decorated particular carriers as a drug delivery system by enzymatic processing, such as protease. (Modified from Jiang et al. (2004))

decrease unspecific bindings of these delivery systems to the negatively charged components of the endo- and epithelium, leading to a favorable lower volume of distribution. An activatable CPP approach to deliver far-red fluorescent dyes to cancer cells elicited tenfold increased signals compared to not cleavable controls in mice xenografts (Jiang et al. 2004). Therefore, similar strategies could be used to decorate the nanoparticle surface and to unprotect charged underlying absorption enhancing or targeting sequences by protease activity in specific absorption regions or diseased tissues, respectively, in the future.

In consequence, strategies to direct drug delivery systems to IECs (e.g., enterocytes, M-cells, goblet, or dendritic cells) using targeting moieties such as transferrin, immunoglobulins, vitamins, or lectins; overcoming mucus gel barrier by enhancing adhesion or penetration; avoiding efflux pumps; and using bioresponsive molecules as constituents of delivery systems are several mechanisms, alone or in combination, which may lead to an enhanced penetration and permeation through intestinal epithelium and then reach the systemic circulation.

9.5 Toxicology of Nanoparticles

Given the advances of nanotechnology applied to medical sciences, a good comprehension of the toxicological profile of nanoparticles is needed. This is justified by the difference in behaviors between nanoparticles and the bulk material (Mortimer and Minchin 2017). Therefore, nanotoxicology has emerged as "the study of the adverse effects of engineered nanomaterials on living organisms and the ecosystems, including the prevention and amelioration of such adverse effects" (Hobson and Guy 2014).

9.5.1 Toxicological Pathways

Several toxicological mechanisms have been described for nanoparticle toxicity. These particles can produce reactive oxygen species and therefore, cause oxidative stress in cells, followed by DNA damage and apoptosis, resulting in many pathological conditions, including cancer, diabetes, and cardiovascular diseases (Khanna et al. 2015; Oberdörster et al. 2005). Oxidant production has been associated with a direct mechanism catalyzed by nanoparticles and the leached ions, by Fenton and Haber–Weiss reactions (Mortimer and Minchin 2017; Yan et al. 2013). Besides, an indirect pathway has been proposed and consists of disturbance of reactive oxygen species-related equilibrium, including disruption of the electron transport chain in mitochondria and activation of enzymes and receptors associated with oxidants production (Yan et al. 2013). This mechanism of toxicity is relevant for metal oxide nanoparticles, including titanium dioxide (Halamoda Kenzaoui et al. 2012; Shi et al. 2013), iron (Halamoda Kenzaoui et al. 2012; Khan et al. 2012), and cerium oxides (Alili et al. 2013; Park et al. 2008). However, there is evidence that also suggests that carbon materials (Liu et al. 2011; Muthu et al. 2013) and liposomes (Zhong et al. 2013) can induce oxidative stress.

Inflammation is another mechanism of nanoparticle toxicity and is triggered by the interaction of particles with cell surface receptors, causing oxidative stress and activation of inflammation after the uptake process (Mortimer and Minchin 2017). An important step for inflammatory process activation is opsonization. This opsonized-nanoparticle complex is recognized by several types of cell surface receptors, including macrophage-1 antigen and toll-like receptors (Khanna et al. 2015; Mortimer and Minchin 2017). Both cell surface recognition and the oxidative process can induce the synthesis of nuclear factor κB (NF-κB) and therefore, the release of other proinflammatory cytokines (Khanna et al. 2015; Mortimer and Minchin 2017). After nanoparticle internalization, a multimeric protein complex is formed, so-called inflammasome (Mortimer and Minchin 2017). This structure triggers host defense by the production and activation of caspases, leading to the release of proinflammatory cytokines (Latz et al. 2013; Man and Kanneganti 2015). As seen for oxidative stress, metal nanoparticles are prone to trigger inflammation effects in cells and eventually cell death (Deng et al. 2017; Kennedy et al. 2009; Lee et al. 2009).

Genotoxicity and cytotoxicity are the other pathways of nanoparticle-related toxicity. Several kinds of particles can induce genetic damage, including DNA strand breaks, mutations, and chromosomal aberrations (Mortimer and Minchin 2017). Metal nanoparticles, including palladium (Alarifi et al. 2017), silver (Wen et al. 2017), titanium dioxide (Shukla et al. 2011), and zinc oxide (Valdiglesias et al. 2013), have been related to DNA damage in animal and human cell models, causing by oxidant formation. Paget et al. have demonstrated that aminated polystyrene nanobeads induce genotoxicity and cytotoxicity in Calu-3 and THP-1 macrophage cell cultures (Paget et al. 2015). Arising from metallic nanoparticles, an important

issue to note is that a nanoparticle may cause toxicity by different pathways at the same time.

9.5.2 Toxicological Determinants of Nanoparticles

As seen in Sect. 9.3, physicochemical properties of nanoparticles are considered as important characteristics for the design and function of nanomaterials as delivery systems. However, these parameters also determine the toxicity of nanoparticles.

The particle size is relevant for enhancing the cell internalization process, leading to increased accessibility of nanoparticles to cells and tissues. Also, the biodegradability of these particles is an important determinant, due to nonbiodegradable nanoparticles may accumulate in the cell and trigger harmful or deleterious effects (Chatterjee et al. 2017). Prietl et al. have demonstrated that 20 nm carboxyl polystyrene nanoparticles were cytotoxic to leukocytes, monocytes, and macrophages, associated with IL-8 production-induced oxidative stress; however, 500 and 1,000 nm-sized nanoparticles only elicited cytotoxic effects on macrophages, related to oxidative stress induced by IL-6 and IL-8 secretions (Prietl et al. 2014). Another important aspect related to the particle size is pharmacokinetics. Nanoparticles with a diameter lesser than 6 nm can be excreted by the kidneys, but larger counterparts cannot, and accumulation in specific organs is observed, such as the liver and spleen, and can cause cytotoxicity (Shin et al. 2015).

The shape is another determinant parameter of nanoparticle toxicity, influencing the physiological responses. Zhang et al. have shown that needle-shaped PLGA-PEG nanoparticles induce human liver carcinoma cell death, by lysosome disruption, DNA fragmentation, and apoptosis, compared with spherical-shaped (Zhang et al. 2017). Indeed, both particle size and shape are relevant to determine the total surface area, an important parameter of particle toxicity, due to the increased chance of contact and interaction between the particle and cell surface, leading to an enhanced cell internalization (Mortimer and Minchin 2017). Therefore, nanoparticles with larger surface areas may lead to increased reactivity and toxicity, associated with higher production of reactive oxygen species, DNA damage, or mitochondrial perturbation (Chatterjee et al. 2017; Shin et al. 2015).

Surface charge is important for interaction with the cell membrane and therefore, the rate of cell internalization process. Charged nanoparticles are prone to produce cytotoxic effects more than neutral counterparts, and positively charged particles tend to elicit higher toxicity than negatively counterparts (Fröhlich 2012; Shin et al. 2015). Naha et al. have demonstrated that positively charged polyamidoamine dendrimers caused cytotoxicity in a mouse macrophage cell line model, related to increased production of inflammatory cytokines and subsequent oxidative stress. Furthermore, as higher positively charged dendrimers, increased toxicity was observed (Naha et al. 2010). Indeed, cationic nanoparticles have elicited to affect cell proliferation, differentiation, and pro-apoptotic genes activation in a human epithelial cell model (Vega-Villa et al. 2008, p. 929–938). In another study conducted

by Mura et al., three types of PLGA nanoparticles have been synthesized and conjugated with different polymers, positively charged chitosan, neutral charged poloxamer 88, and negatively charged poly(vinyl alcohol). All these nanoparticles have demonstrated very limited cytotoxicity with no inflammatory response in Calu-3 cells model (Mura et al. 2011). The low cytotoxic effect of positively charged-conjugated PLGA nanoparticles is associated with the biocompatibility of natural chitosan (Fröhlich 2012). A good strategy to reduce cytotoxicity of cationic particles is using PEG as a coating agent, as demonstrated by Luo et al. that showed PEGylated polyethyleneimine-DNA complexes reduce cell death in HeLa cells model and even improve the transfection efficiency (Luo et al. 2012).

Another important toxicity determinant is surface coating agents. Some polymers used as surface coating agents may cause some deleterious effects, affecting the chemical reactivity and cytotoxicity of nanoparticles. Indeed, these agents play an important role in favoring the contact between particles and the cell surface (Chatterjee et al. 2017). Grabowski et al. have demonstrated that chitosan, poly(vinyl alcohol), and poloxamer 88, used as stabilizers in PLGA nanoparticles, caused cytotoxicity in human-like THP-1 macrophage model, while nonconjugated PLGA particles have elicited no toxic effects in the same cell model (Grabowski et al. 2015). Other commonly used surface coating agents are surfactants. Wang et al. have realized that cetyltrimethylammonium bromide is responsible for cytotoxicity elicited in the human skin keratinocyte model by surfactant-coated gold nanoparticles (Wang et al. 2008). Similarly, Zhang et al. have demonstrated the cytotoxic effect of long-chain cationic and anionic surfactants (Zhang et al. 2016). Therefore, the same physicochemical properties that are relevant for cell internalization of nanoparticles, i.e., particle size, shape, charge, surface properties, and degradability, are related to the toxicity profile and may produce deleterious effects, including oxidative stress and potential cell death.

9.5.3 Systemic Toxicity of Nanoparticles

As described above, the oral route is the most acceptable and tolerated route of administration for drugs and drug delivery systems. Oral toxicity of nanoparticle has been associated with several types of particles, mainly by direct ingestion of these drugs or drug delivery systems, but nanoparticles can also reach the gut from ingestion-induced by inhalation (Dobrovolskaia et al. 2017; Vega-Villa et al. 2008). Indeed, nanoparticles can enter the bloodstream by oral absorption and be distributed throughout the organism and accumulate in cells and tissues, leading to systemic toxicity (Dobrovolskaia et al. 2017). Therefore, the toxicity profile of nanoparticles is required for a good comprehension of the behavior of these drug delivery systems.

The most described systemic toxicity induced by oral administration is related to metal oxide nanoparticles. Inflammatory responses have been reported in a repeated dose administration of silver particles in mice model, related to increased production of cytokines (Park et al. 2010). Park et al. have demonstrated that after 90 days

of oral administration of 20 nm-negatively charged zinc oxide nanoparticles in Sprague Dawley rats elicited a decrease in several anemia-related hematological and blood biochemical parameters related to the accumulation of zinc; and also pancreatic acinar cells apoptosis and stomach lesions induced by continuous irritation of orally administered particles (Park et al. 2014b). Similar results were obtained using 20 nm positively charged zinc oxide nanoparticles (Park et al. 2014a).

For lipid-based nanoparticles, such as liposomes and micelles, toxicity profile is associated with lipid composition, particle size, and charge (Sharma et al. 2012). Cationic liposomes have been used as possible carriers for gene therapy; however, the positively charged nanoparticles have been related to the cytotoxic effect, by interaction and inhibition of critical enzymes, such as protein kinase C (Lv et al. 2006). Knudsen et al. have performed an in vivo rat model assay and determined that cationic micelles and liposomes increased DNA strand breaks in the lung and spleen and liposomes induced more toxicity than micelles (Knudsen et al. 2015). Roursgaard et al. have demonstrated the same results in an in vitro model using human hepatocyte and lung epithelial cell lines (Roursgaard et al. 2016). These results are related to the cationic hydrophilic group of lipids and nanoparticle accumulation in mononuclear phagocyte system organs, after administration and systemic absorption (Sercombe et al. 2015). Therefore, the chemical nature and composition of these lipid-based nanoparticles are important considerations in the formulation and elaboration processes.

Polymer-based nanoparticles have been related to minimal or noncytotoxic effects. For example, PLGA is an FDA-approved polymer for the synthesis of drug delivery systems due to polymer biodegradability and biocompatibility and has demonstrated an enhanced accumulation of drugs in targeted tissues (Sharma et al. 2012). Chitosan is a positively charged polysaccharide-derived polymer and has been used to increase paracellular permeability, mucoadhesive property, and cellular internalization of nanoparticles (Bowman and Leong 2006). As PLGA, chitosan also elicits relevant features, such as biodegradability and biocompatibility (Rodrigues et al. 2012); however, a high degree of deacetylation, i.e., the proportion of glucosamine and *N*-acetyl-glucosamine that determine the total positive charges, elicited greater cytotoxicity; thus, a 65% of deacetylation is considered as an optimal value to prevent this toxic effect (Bowman and Leong 2006). Ojer et al. have demonstrated that uncoated, 2-hydroxypropyl-β-cyclodextrin-coated, and PEG 6000-coated poly(anhydride) nanoparticles have no toxic effects in Wistar rats after oral administration (Ojer et al. 2012). Similar results have been obtained by Syama et al. using dextran-coated ferrite nanomaterials in an in vivo model after oral administration (Syama et al. 2014). However, some deleterious effects have been reported for dextran, including anaphylaxis, pulmonary edema, and platelet dysfunction (Sharma et al. 2012).

An issue that is important to describe for any drug and drug delivery system is immunotoxicity. The nanoparticle-related toxic effects on the immune system are related to particle interactions with serum proteins and sites of particle accumulation (Dobrovolskaia et al. 2017). There are several mechanisms that nanoparticles can alter the induction of immune responses: by delivering antigens, accumulation

effect, repetitive exposures, and cross-immune reactions (Smith et al. 2013). Although coating with PEG is a strategy commonly used to enhance circulation time and reduce an immunogenic response for nanoparticles, some evidence established that this agent may be immunogenic and induce the production of anti-PEG antibodies, associated with an increase of blood clearance and diminished efficacy of these drug delivery systems (Schellekens et al. 2013). Indeed, Mima et al. have demonstrated that anti-PEG IgM is the major determinant of these immune responses (Mima et al. 2015). Recent findings have determined that the occurrence of anti-PEG antibodies in healthy blood donors is ranged from 22% to 25%, due to greater exposure of PEG-containing cosmetics, drugs, and food products (Garay et al. 2012). The study of Wan et al. has demonstrated that the extent of PEGylation and the molecular weight of methoxyPEG are relevant for immunotoxicity, while branching of methoxyPEG had no significant effect in immune response (Wan et al. 2017). Another mechanism of immunotoxicity is related to the formation of protein corona at the particle surface. This protein corona is composed of several serum proteins, including IgG, albumin, fibrinogen, and apolipoproteins, and may trigger an immune response and affect the nanoparticle toxicity profile and targeting properties (Corbo et al. 2016). Therefore, the systemic and immune toxicity studies must be needed for a better determination and characterization of the safety profile of nanoparticles.

9.5.4 Regulation Aspects of Nanoparticles

Given the rise and advancement of nanotechnology applied to the medical sciences in recent years, engineered nanomaterials must be regulated, according to the safety concerns. Thus, nanoparticles are considered as chemical particulates and regulated by Toxic Substances Control Act (TSCA), depending on the US Environmental Protection Agency, and by Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), related to the environmental subdivision of the European Commission (Dobrovolskaia et al. 2017). Furthermore, nanoparticles designed for medical applications or used as medical devices (e.g., drug delivery systems) are regulated by the US Food and Drug Administration and European Medicines Agency (Dobrovolskaia et al. 2017; Hobson and Guy 2014). The aspect of regulation for nanomaterials was first incorporated by European Union and Switzerland into the existing legislation and is reviewed in detail in other publications (Amenta et al. 2015; Rauscher et al. 2017). In general, these and other regulatory entities are focus on the safety issues of nanoparticles, including the development of good practices, risk assessment, exposure evaluation and mitigation, control over safety, and sustainable use of nanotechnology (Dobrovolskaia et al. 2017).

9.6 Conclusions

Nowadays, medicine and health care have evolved according to the progress and development of new technologies and strategies to diagnose and treat several diseases. Using the advance of nanotechnology applied to this field, there are multiple knowledge and evidence that nanoparticles offer several advantages and may represent a big tool as drug delivery systems, especially for orally administered poorly water-soluble and permeable drugs. Therefore, the design and development of an optimal nanoparticle used as an oral drug delivery system may serve as a tool to overcome the intestinal epithelial barrier and should consider the anatomical and histological features of the gastrointestinal tract. Furthermore, several physicochemical characteristics of designed nanoparticles, including size, shape, surface charge, chemical composition, and particle deformability and degradability, are important for the understanding of the mechanism and rate of internalization process through the intestinal epithelium and also relevant for the potential role in the toxicity of these nanosized drug delivery systems. To enhance the internalization rate in IECs for poorly water-soluble and permeable drugs after oral administration, there are several strategies that could be used to achieve this purpose, including specific IEC targeting, overcoming the mucus layer, and avoiding efflux pumps. Indeed, there are several mechanisms that could be exploited by the distinct physiological conditions of this route of administration, including pH and digestive enzymes, and even more the distinct pathological conditions, such as cancer or inflammation, using bioresponsive delivery systems. Therefore, comprehensive knowledge of these topics may conduct a rational design and elaboration of an optimal oral drug delivery system.

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Chapter 10
Recent Progress in Nanotheranostic Medicine

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Contents

Abstract Issues: In the area of regenerative medicine paradigm, theranostic nanomedicine is emerging as a promising prototype. It has the advantage of both imaging and therapeutic functions in the platform of personalized medicine. Recently scientists have developed smart and hybrid forms of biological molecules, as carriers for various applications in diagnostics and therapeutics.

Major Advances: We reviewed the advances on nanotheranostic materials. Interactions among the particles in carrier payload have critical roles in the efficacy of a drug in clinical translation. Cancer and other degenerative diseases have remained indomitable problems for scientists and doctors due to their diverse nature and etiology. Nanotheranostic agents are now emerging as prudent tools in the detection of diseases, especially cancer. In recent years, the utilization of inorganic

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particles like iron oxide, gold, and carbon dots has gained massive attention in imaging technologies like MRI, PET, and SPECT. However, cumulative information on nanotheranostic agents, such as nature of materials, cell interaction, toxicity, mode of action at cellular levels, and effects on microenvironmental milieu, are required. Finally, we discuss the progress of different types of theranostic agents, their modes of biodistribution, pharmacokinetic properties, and chemocellular interactions.

Keywords Nanoceutical · Nanotheranostic · Biomaterial · Regenerative medicine

Abbreviations

CAT	Computed axial tomography
CT	Computed tomography
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
HIFU	High-intensity focused ultrasound
IMPT	Intensity modulated proton therapy
IMXT	Intensity modulated x-ray therapy
LHRH-PE40	Luteinizing hormone-releasing hormone-*Pseudomonas aeruginosa* exotoxin 40
miRNA	Micro-ribonucleic acid
MNP	Magnetic nanoparticles
MRI	Magnetic resonance Imaging
NP	Nanoparticles
PET	Positron emission tomography
SDT	Sonodynamic therapy
SELEX	Systematic evolution of ligands by exponential enrichment (SELEX) method
SiRNA	Small interfering ribonucleic acid
SPECT	Single-photon emission computed tomography

10.1 Introduction

Nanotheranostic, an advanced branch of nanomedicine, is an amalgam of both diagnosis and therapeutics with fewer side effects, even for stubborn pathological conditions (Kojima et al. 2015). The strategy of nanotheranostic involves simple biological probing with easy monitoring of targeted drug delivery with more stability under physiological conditions (Sahoo et al. 2014). Nanotechnology-based theranostics approach is based on manipulating nanoparticles (NPs) (1–200 nm) by exploiting their unique properties, such surface area, optical and magnetic properties, low

melting point, and mechanical strength (Horikoshi and Serpone 2013). A theranostic agent consists of a delivery platform that is conjugated either covalently or non-covalently to a therapeutic drug, nucleic acid (miRNA, siRNA), therapeutic proteins, or any other chemotherapeutic agent and signal emitters (with unique radioactive, optical, or magnetic properties) (Baum and Kulkarni 2012). Gold, silver, and magnetic NPs, along with nanoshells and nanocages, are important nanotherapeutical agents, that can be conjugated to either drugs, ligands, or antibodies to enhance the delivery and therapy as well as diagnostic imaging of the stages of diseases (by MRI, CT scan, CAT scan ultrasound, etc.) (Mody et al. 2010). The central principle and multimodal application of different nanotheranostic agents is shown in Fig. 10.1. It is clear from the model that the theranostic principle can be effectively utilized for a variety of activities in the area of biomedical, bioengineering, and biotechnological field. Therapeutic effectiveness of various drugs along with diagnostic agents has emerged as a future disease management system. Centrally, therapeutic drug and carriers are surface conjugated in a nanoform for various diagnostic purposes in the area of biomedical and biotechnological applications by bioengineering designs. Such an artificially designed drug for reliable diagnostic as well as therapeutic purpose enhances the efficacy of drug, especially in the area of cancer treatment. Cooperative joint delivery of drug and diagnostic agents could pave way to new insights for management of various degenerative diseases (Tinwala and Waikar 2019). This intelligent and smart revolutionary synthetic chemistry along with engineering tools is emerging as a powerful platform for modern diseases like cancer, vascular disease, and diabetic. Intracellular biostability, biocompatibility, low toxicity, and point of target are major advantages of this theranostic technology. Therefore, it is gaining importance in modern medicine. Accordingly, many varieties of nanocarriers for efficient conjugation have been developed in the recent past.

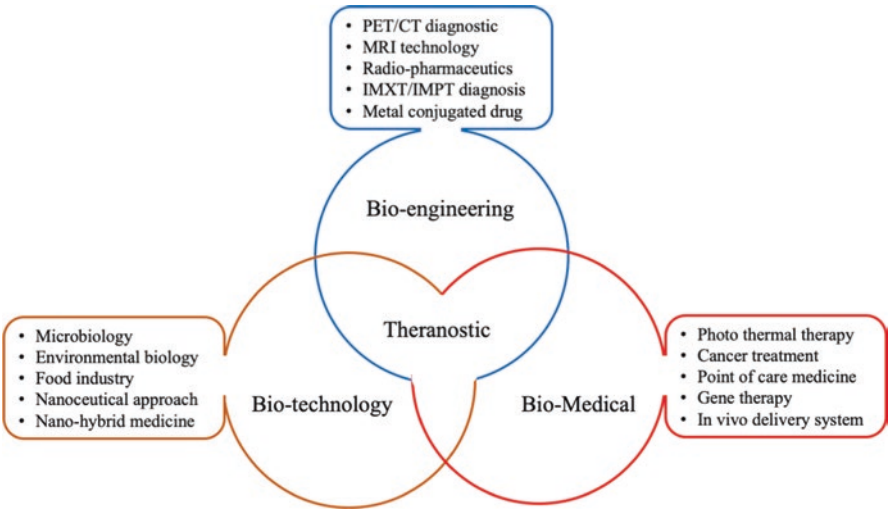

Fig. 10.1 Theranostic technology in applied biological fields like Bioengineering, Biomedical and Biotechnology

Table 10.1 Promising theranostic agents and its targets

Materials	Target cell/organ/tissue/field	Reference
Transferrin-stabilized nanomagnets	Human fibroblast	Berry et al. (2004)
Magneto-dendrimers	Stem cells	Bulte et al. (2001)
CEA–maghemite conjugate	Colorectal cancer	Campas de Paz et al. (2012)
Supermagnetic iron oxide	Schwann cell in CNS	Dunning et al. (2004)
Hybrid gadolinium oxide	Blood circulation pattern	
Fluorescent magnetic nanoparticles	Mouse whole body	Kwon et al. (2008)
LHRH–magnetic iron oxide particles	Breast cancer cells	Leuschner et al. (2006)
Carbon nanotube	Mouse tumor	Liu et al. (2007)
Octavalent peptides	Tumor imagining	Luo et al. (2012)
Dextrin-coated monocrystalline iron oxide	Tumor cells and macrophages	Moore et al. (1997)
Biobarcoding system	Protein theranostic	Nam et al. (2003)
Magnetic nanoworms	Tumor imaging	Park et al. (2009)
Aptamer cell–SELEX	Liver cancer	Rong et al. (2016)
Magnetofection	Gene delivery	Scherer et al. (2002)
Silica-coated material	Lung cancer	Tartaj et al. (2001)
Magnetic nanoparticle	Central nervous system	Tomitaka et al. (2019)
Polyethylenimine and folic acid	Cancer biology	Zhang et al. (2002)
Graphene	Cancer biology	Orecchioni et al. (2015)

For example, protein-based NPs and synthesized nano-scale DNA three-dimensional (3D) lattice structures (DNA origami) have been used as theranostic agents where various chemotherapeutic drugs compartmentalized into the hollow spaces inside the lattice can enhance the efficacy of controlled drug delivery and cancer biomarkers (Pinheiro et al. 2011). These nanotherapeutics can also be used for targeted delivery approach with real monitoring of both drug release and biodistribution of the NPs with facilitation of biomedical applications, such as efficient drug delivery across the blood-brain barrier (Tomitaka et al. 2019), multimodal and combinatorial therapies, and co-delivery of antisense oligonucleotides (siRNAs) (Muthu et al. 2014). The NPs can deliver a variety of targeting agents such as peptides, aptamers, monoclonal antibodies, nucleic acids, chemotherapeutics, etc. to malignant cells due to their surface modifications, enhanced permeability, and retention effect (Baetke et al. 2015). Different and promising types of nanotherapeutic agents with properties and target molecule are mentioned (Table 10.1).

10.2 Nomenclature of Nanotheranostic Material

Nomenclature of the nanotheranostic agents is principally based on the arrangement and organization of different drugs, theranostic agents, and target molecules. All three principal molecules are designed with the help of a linker molecule. There are

several types of theranostic agents. Linear types have end positions attached to target agents. In polymeric and solid-phase types, diagnostic and theranostic agents are immobilized with linker-assisted target agents. In dendrimer type theranostic material, the endpoint of each branch is conjugated with detecting theranostic and drug molecule. In lipid-based types like liposome- and noisome-based types, micelle compounds are synchronized with diagnostic and theranostic materials. Finally, gold or any metal and carbon-based theranostic agents are prepared by simple chemical reactions. Different base materials are used to synthesize each of these types. Magnetic nanotheranostic is one important and widely used nanotherapeutical, due to its multipurpose functions such as hyperthermal killing of cancer cells, efficient delivery of gene and conventional chemotherapies, as well as multimodal imaging (PET, MRI, and optical imaging) (Singh and Sahoo 2014). Theranostic, coupled with MRI, have various advantages like free ion scanning mode, a high degree of tissue penetration with noninvasive detection mode. Due to their small size, they not only offer a better tissue penetration and faster drug delivery but also can provide a better platform for a diverse array of modifications with chemotherapeutic and target moieties (Xie et al. 2011). They are now used as contrast agents in MRI for diagnosis of cancer cells in soft tissue due to low cytotoxicity (Yoo et al. 2013). It has been shown that the superparamagnetic IONPs (SPIONs) loaded with amphiphilic poly(styrene)-b-poly(acrylic acid)-Dox and folic acid can be used as potential theranostic agents, as demonstrated in SkBr3 (human breast cancer cell) and HCT116 (human colon cancer) cell lines for controlled release, targeting, and anticancer activity (Patra et al. 2014).

Gold- and silver-based nanotheranostic materials are gaining importance in recent years due to their wide application ability in cancer biology. Gold and silver NPs are promising nanotheranostic agents due to their easy synthesis, bioconjugation, and surface modification (Boisselier and Astruc 2009). Use of gold nanoparticles in the diagnostic arena has attained considerable interest in biomedical, agricultural, and environmental including forensic investigation due to availability of highly advanced optical method, namely, surface-enhanced Raman spectroscopy (SERS). Due to laser excitation on gold-labeled drug surface in the SERS analysis, it has a high degree of application in MRI technology in tumor identification. These are used for multimodal imaging as well as to combat different types of cancer by exploiting its efficacy on cancer cell local effect. Cancer cell drug target is focused on the various intracellular cancer cell microenvironments like redox potential, ROS status, and cellular pH. The schematic of gold-based plasmonic SERS system is explained in Fig. 10.2.

Gold as plasmonic materials has been widely used for developing SERS active sensors for intracellular targets. Due to its high specificity, enhanced cellular uptake, and negligible cytotoxicity, gold nanobeacons (AuNBs) are used for specific mRNA silencing (Baptista 2014). Gold nanoclusters (AuNCs) conjugated with chitosan biopolymer are used for imaging due to its properties like low photobleaching, negligible cytotoxicity, and enhanced Stokes-shifted emission (Sahoo et al. 2014). Graphene-based nanotheranostic compounds are recently gaining much attention as inert and nonevasive compounds. Due to their large surface area, colloidal stability,

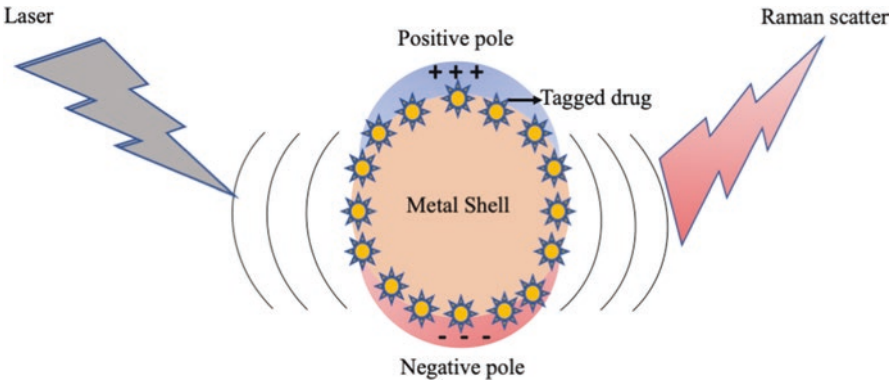

Fig. 10.2 The schematic of gold-based plasmonic SERS system

easy surface modification/functionalization), as well as superior electrical and mechanical properties, graphene oxide-based nanomaterials provide significant attention in image-guided molecular ablation of cancerous cells (Draz et al. 2014). With a combination of AuNP, graphene-based nanotherapeutic agents are used in phototherapy; GO-Au-IONP assemblies have been shown to have enhanced superparamagnetism, optical absorbance, and photothermal therapeutic potential (Shi et al. 2013).

Due to distinct properties such as chemical and thermal stability, large surface area, and pore volume, silica NPs (SiNPs) function as an important theranostic agent which can be used for controlled, sequential, and multifunctional delivery drugs to numerous cancer cell types (Draz et al. 2014). Silica nanorattles conjugated with LHRH-PE40 luteinizing hormone-releasing hormone-*Pseudomonas aeruginosa* exotoxin 40 and docyanine green fusion protein can be used as a nanotheranostic agent in combination with docetaxel (antimitotic chemotherapeutic) for treatment of cancers (Gao et al. 2013). These days, lipid- and polymer-based nanotheranostic agents, mainly liposomes, dendrimers, and polymeric micelles, are widely used as nanocarriers due to their easiness for surface modification, targeting ability, thermal stability, and compatibility with various diagnostic applications (Gu et al. 2007). They have a wide range of capability, such as cellular compatibilities, biodegradability, rapid cellular uptake, and lack of toxicity (Schroeder et al. 2010). Protein-based nanotheranostic agents are made up of proteins which can be used as carriers for both therapeutic and diagnostic agents (Ng et al. 2011). These nanocages can be modified both internally such as loading with conjugable molecules (drugs/aptamers/contrast agents) and externally by ligand conjugation (Lim et al. 2013, Kaur and others 2018). Other classes of protein-based nanotheranostic agents such as nanoradiopeptides and fluorescent peptide nanoprobes (Luo et al. 2012) also hold immense promise as major therapeutic agents for cancer treatment. Targeted binding of ions and small molecule proteins with artificially synthesized RNA or DNA oligonucleotides also called aptamers through a process called systematic evolution of ligands by exponential enrichment (SELEX) method (Tuerk

and Gold 1990) have effectively been used in the cell-based theranostic procedure (Kaur 2018). Due to the wide angle of advantage, SELEX has been widely used against cancer cells, tumor-associated proteins, and parasites or virus-infected cells (Rong and others 2016).

10.3 Biodistribution

Biodistribution of the nanotheranostic agents depends upon various factors such as size and shapes. For example, hybrid gadolinium oxide NPs (Gado-6Si-NP) were selectively taken up by circulatory blood pool and finally cleared by renal excretion without accumulation in the liver. The effectiveness of this particular theranostic agent is due to its small size of 3–4 nm diameter (Kryza et al. 2011). The strategy of application of theranostic agents in cancer biology and detection method is depicted in Fig. 10.3. The fate of cancer cell growth or death can be monitored through surface modification of theranostic agents using smart, radiowave-emitting or fluorescent tags that are cancer cell-specific and capturing these signals either by amplifier-assisted receiver or appropriate fluorescence detection instrument. In vivo barriers like cell membrane and blood–brain barriers are major checkpoints for NP movements in the body, by restricting the NP functions like their movement and physical changes and inducing a negative host response (Belting et al. 2005). Blood,

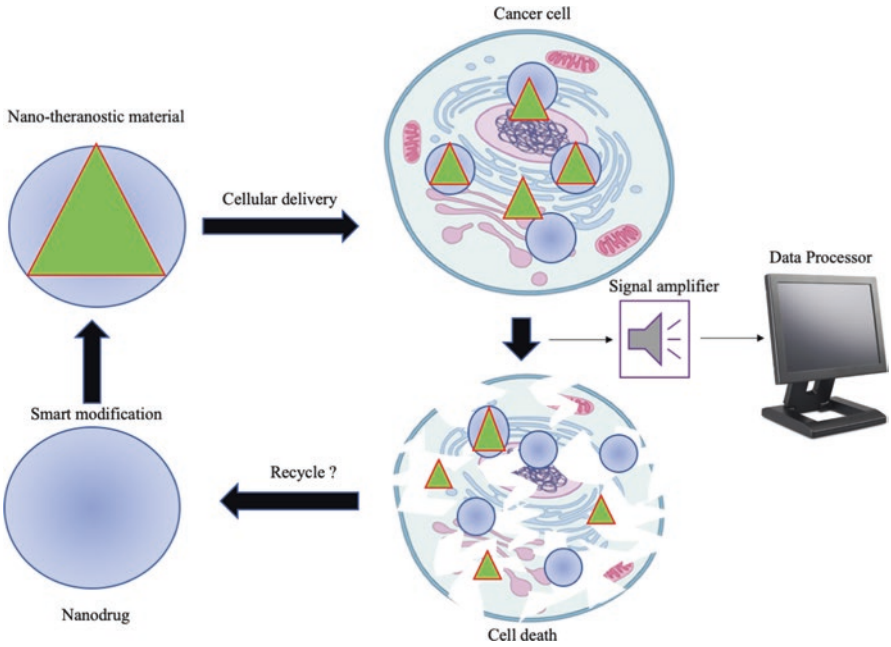

Fig. 10.3 The strategy of application of theranostic agents in cancer biology and detection method

its components, anatomical restriction, and different barriers like blood–brain barrier are important components through which the NPs have to pass. Effective traffic control and distribution in the body are key strategical factors for the optical design of NPs. Smart design is an important factor for the biodistribution of nanotheranostic agents through the biological barriers and enabling them to reach their intended destination (Ferrari 2005).

The physiochemical properties, which include morphology, hydrodynamic size, charge, and other surface properties, are important factors for the biodistribution of nanotheranostics (Dobrovolskaia et al. 2008). One of the important physicochemical properties is the hydrodynamic size. Hydrodynamic size helps in governing the NP concentration in the blood vessel by affecting the mechanism of NP clearance and dictates the permeability of NPs out of the vasculature (Chavanpatil et al. 2006). It also affects the NP clearance from the circulation and also determines the passage of it through the blood–brain barrier (Koo et al. 2006)**.** Another aspect of the physicochemical nature and action of a theranostic agent is governed by the shape (Gratton et al. 2007). It was found that the anisotropically shaped NPs can avoid bioelimination better than spherical NPs (Liu et al. 2007). Some scientists also have shown that high aspect ratio shaped MNPs have also been evaluated in vivo and found to have similarly enhanced blood circulation times over the spherical counterparts (Park et al. 2009). Surface properties also play important roles in the target action of theranostic agent because major agents are targeted toward the cell receptors. Surface properties such as NP charge and hydrophobicity can affect biodistribution by minimizing or enhancing the interactions of NPs with the adaptive immune system, plasma proteins, extracellular matrices, and nontargeted cells (Davis 2002). These two factors mainly contribute to the short circulation time, which may be due to the adsorption of plasma proteins recognized by the reticuloendothelial system [RES] (Chouly et al. 1996)**.** Target designed specificity is yet another important aspect to be considered during designing of the theranostic agent. For both diagnostic imaging and drug-based therapies for selected tissues, the specificity of NPs is an important factor for successful nanotheranostics (Leuschner et al. 2006). NPs have been engineered to have an affinity for target tissues either through passive, active, or magnetic targeting approaches. Passive targeting uses the predetermined physicochemical properties of a given NP to specifically migrate to a given tissue region by the phenomenon known as enhanced permeation and retention (EPR) (Maeda et al. 2000). Toxicological preevaluation of NP is an important aspect for end application (Madhyastha et al. 2019). In general, the toxicity of the compound to be conjugated and NP should be considered before development of an appropriate therapeutic agent. It mainly involves considering how the assembled NP will interact with the body and how the independent components will affect the body during biodegradation and liver processing (Lewinski et al. 2008). This leads to a new branch that is nanotoxicology, which mainly deals with an understanding of the body's response to the NPs' chemistry (Vega-Villa et al. 2008).

10.4 Pharmacokinetics

Nanotheranostic agents undergo typical pharmacokinetics pathway in which they are clustered within lysosome upon their intracellular internalization via endocytosis. They are degraded into corresponding metal ions by an array of hydrolyzing enzymes at low pH (Gupta et al. 2007). The size, charge, surface chemistry, and route of delivery influence the circulation time and biodistribution pattern inside the body. The spleen is the organ in which the large particles are usually sequestered and small particles are rapidly removed through extravasations and renal clearance upon intravenous injection. (Gupta and Gupta 2005). The final distribution of the particles is observed more in the liver (80–90%), followed by spleen (5–8%), and less in bone marrow (1–2%) which mainly depends upon the surface chemistry and the mechanism of internalization (Unfried et al. 2007). NPs may interact with the extracellular matrix components and the plasma cell membranes of macrophages, endothelial cells, skin epithelium, and respiratory or gastrointestinal tracts during their metabolism (Oberdorster et al. 2005). Upon inhalation, they are accumulated in the brain, liver, spleen, and lungs demonstrating their ability to cross blood–brain barrier (Kwon et al. 2008). Macrophage plays an important role in nanotheranostics clearance. Nanotheranostic agents are challenged by macrophages of the RES upon their administration in vivo (Duguet et al. 2006). Many mechanisms of the internal organization such as phagocytosis (mediated by mannose, complement, Fcγ, and scavenger receptors), endocytosis (clathrin- and caveolin-mediated, fluid-phase), and diffusion are involved for processing of nanotheranostics in macrophage (Dobrovolskaia and McNeil 2007; Unfried et al. 2007). NPs can also get opsonized by plasma proteins (e.g., albumin, apolipoprotein, immunoglobulins, complement, fibrinogen), which promote their recognition and clearance by cells of RES (Park et al. 2008). Iron oxide Nano Particle (IONP) binds to the plasma fibronectin, and vitronectin changed from receptor-mediated to fluid-phase endocytosis (Moore et al. 1997). The polyethylene glycol (PEG), which acts as amphiphilic polymeric surfactants, significantly reduces MNP interactions with plasma proteins, minimizing their internalization and clearance by macrophages (Zhang et al. 2002). PEG along with antitumor drug paclitaxel is being investigated in metastatic breast cancer xenograft mouse model and is found to be effective in reducing the tumor size over the period of administration (Lee et al. 2018), since cancer cell has several receptors for PEG and also free-circulating nature of PEG in bloodstream resulting in remarkable antitumor efficacy.

10.5 Surface Functionalization

Figure 10.4 depicts the various types of nanotheranostic agents by smart designing approach. Linear-type polymer and drugs are conjugated in an exponential manner. Target capture agents are capped at each corner of the polymer edge. Other types are

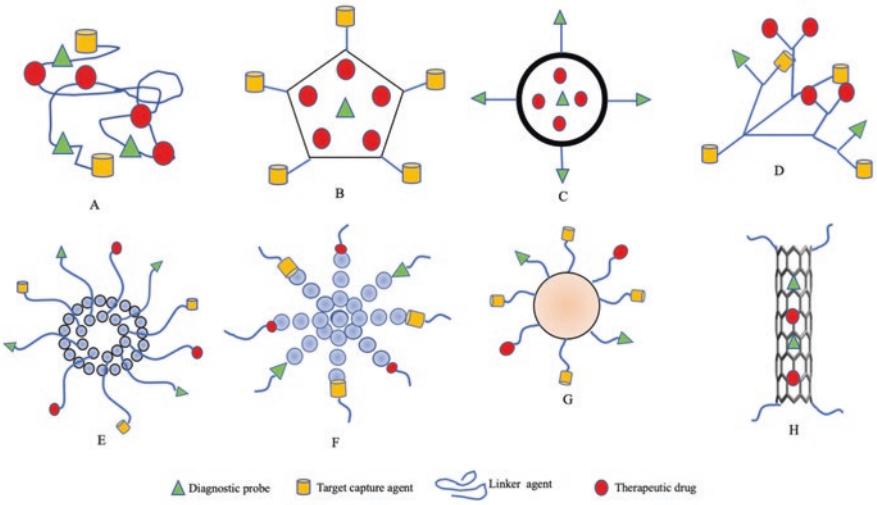

Fig. 10.4 The various types of nanotheranostic agents by smart designing approach

of solid-phase, dendrimer, liposome, and noisome types. In metal variety, gold or silver metals are centrally placed. Surface functionalization, which increases the surface activity and biocompatibility, is an important biological phenomenon needed for all the nanotheranostics reagents (Medha et al. 2018). Biodegradable polymers are safe and green materials that have been widely researched by many investigators. Several formulations such as dextrans, chitosan, polyethylene glycol, polysorbate, and polyaniline are used for increasing the surface functionalization of nanotheranostics agents. Dextran is an important constituent for various formulations such as Ferridex, Resovist, Combidex, and AMI-288/ferumoxytol (McCarthy et al. 2007). Polyethylene glycol due to its hydrophilicity and low antigenicity prevents plasma opsonization and uptake by macrophages and thus increasing the theranostics circulation in vivo (Gupta and Curtis 2004). Its excellent film-forming, emulsifying, and adhesive properties can be utilized in targeted drug delivery, tissue engineering, and biosensor technology (Gupta et al. 2007). Lastly, chitosan provides a natural, biocompatible, cationic, and hydrophilic polymer coating, suitable for affinity purification of proteins and magnetic bioseparation (Sasaki et al. 2008). Inorganic metals are prosperous materials with proper surface modifications. Different inorganic metals such as gold and silver are used for the surface functionalization due to their high stability and low reactivity (Eustis and el-Sayed 2006), but the dissimilarity of the two metallic surfaces causes a pitfall for use of the metal as coating agent (Lu et al. 2007). Inorganic oxides like metals too play a governing role in surface chemistry during synthesis and design of theranostic agents. Inorganic oxides such as silica gel can be used as a coating agent due to their negative charge and stability in aqueous solution. Silica-coated magnetic nanoparticles have longer circulation times, and their hydrophilic negatively charged surface provides ideal

anchorage for covalent binding to ligands, presenting an excellent platform for drug delivery (Tartaj et al. 2001). In the area of cancer biology, use of dimercaptosuccinic acid is found to be a suitable agent as a surface modifier. Among all cancer etiology, colorectal cancer is considered as one of the major cancer types with huge mortality worldwide. Cancer of the large intestine (colon, rectum, and anus) is a major cause of morbidity. Nanosized maghemite material precoated with dimercaptosuccinic acid and functionalized anticarcinoembryonic antigen (anti-CEA) is a potent tool in the identification of colon cancer by MRI techniques (Campos da Paz et al. 2012). This system also can be used as a theranostic agent for tumor cells and circulating cancer cells. Surface charges during the synthesis of theranostic agent also play a very important role (Wilhelm et al. 2003). Negatively charged sulfur containing chelating agent prevents the theranostic materials from aggregation and interacts strongly with the positively charged regions of the plasma membrane due to its negative charge.

10.6 Application of Nanotheranostics

Nanotheranostics mainly aims at simultaneous diagnosis and therapy with a wide range of functions in magnetic hyperthermia, drug/gene delivery, tissues engineering, and diagnostic imaging or biosensor platform. For example, the use of magnetic hyperthermia techniques comes under cancer therapy and relies on the localized heating of tumors above 43°c for 30 min (Pankhurst et al. 2003). The magnetic nanoparticle due to its magnetization property can heat the cancer cell, and the selectivity toward tumors was considerably improved through the use of silane coating and through functionalization approaches (Jordan et al. 1999). Tumor growth can be arrested by using magnetic cationic liposomes with a combination approach employing TNF-α gene and stress-inducible gad 153 promoters (Ito et al. 2001). Magnetic resonance imaging is an important diagnostic technique for living tissues in which magnetic NPs are used as contrast agents to identify lymph node metastases and solid tumor (Hogemann et al. 2000). Macrophage-specific MNP labeling protocols are used to image inflammatory pathologies, including atherosclerosis, multiple sclerosis, and rheumatoid arthritis (Berry et al. 2004). MNP-labeled stem cells and neuroprotective glia cells can be guided by in vivo cell tracking of CNS regeneration, while glioma cells are visualized by FITC-conjugated MNPs (Dunning et al. 2004). Theranostic techniques are used to diagnose the rate of bioseparation in vivo (Jian and Rosenberg 2005). Healthy kidney function is evaluated by the rate of glomerular filtration rate. Insulin clearance is the most updated and standard marker for evaluating the glomerular filtrate rate. Alternately, radiolabeled chelating agents like ethylenediaminetetraacetic acid, ^{51}Cr-EDTA; diethylenetriamine pentaacetic acid, 99mTc-DTPA; and radio-iothalamate, 125I-iothalame, are also used in medical pathology. All of these compounds are sensitive to kidney cells and are not cost-effective. Recently iohexol, a

nonradiolabeled, nonionic, low osmolar iodinated agent with very high x-ray contrast properties has shown efficacy as theranostic molecule (Berg et al. 2011).

Drug delivery system is a major concern nowadays for appropriate treatment of different chronic diseases, including various cancers. Current chemotherapy drugs attack both normal as well as cancer cells in the tissue, thus becoming a cause of concern with life-threatening side effects. Targeted drug delivery is a novel approach to overcome this lacuna. This can be done by surface functionalization of different NPs. MNP-conjugated drugs have been applied experimentally for cancer therapy (Duguet et al. 2006). Neurological diseases can be treated with these NP-based drugs and gene delivery system, which can cross the blood–brain barriers (Kreuter 2001). However, for efficient drug delivery, the NPs' surface chemistry, hydrophilicity, and the size are major factors to be considered for the rapid clearance by RES (Torchilin and Trubetskoy 1995). Dual-modality imaging system is comparatively safe, simple, and noninvasive diagnostic and imaging system. But lower image quality is the caveat in this system. Recently magnetic transfection and surface modification of target drug are being used in regenerative medicine. Magnetic transfection is an important application in which NPs are employed as effective transfection system for delivering DNA into the cells. Mainly the MNPs are used for gene introduction to permissive and nonpermissive cells under external magnetic field (Scherer et al. 2002). Effective delivery of antisense oligonucleotide is done through the magnetic field in endothelial cells. (Krotz et al. 2003).

Another hallmark application of theranostic technology is in tissue engineering. During the artificial tissue grafting and developmental studies, varieties of theranostic agents are being used. It is one important application of nanotherapeutics where new tissues are regenerated by using stem cell replacement therapy for cell labeling, sorting, monitoring, and engraftment to the diseased tissue (Bulte et al. 2001). The tissue surfaces are joined under high temperature by using MNPs typically accompanied by protein denaturation followed by repolymerization of adjacent protein chains (Gupta et al. 2007). Keratinocyte sheet-like 3-D constructs have been developed by harvesting MNPs, where self-assembled magnetic nanowire arrays are used (Ito et al. 2005). Another important metal which has been used in theranostic biology is silver. Nanosilver is a major source of antibacterial material because of wide spectrum activity, especially in the wound-healing scenario. Silver, having highest physicochemical properties, very high mechanical strength, and good electrical conductivity, is reported as good sensing as well as imaging agent and is a source of interest for medical and biotechnological research. Silver decahedral NP (Ag10NP) conjugated with fluorophore aptamers (Sgc8-FITC) was found to be the most suitable sensing agent in FRET system (Li et al. 2015). Here target molecule is membrane protein tyrosine kinases-7 (PKT-7) in CCRF-CEM T-cell line. Advantage of using this theranostic agent is to enhance the imaging quality of T cell by disturbing the FERT effect in real-time system.

10.7 Toxicity of Nanotheranostics

Although the theranostic agents display huge advantages, they also exhibit different types of drawbacks like cell stress, cell senescence, and untimely cell death. One of the major concerns of cell stress is oxidative stress. Oxidative stress in the cancer cell is required to cure the cancer; however, the same action in a normal cell can be precancerous. Therefore, fine-tuning between cancer and normal cell is subject for research (Madhyastha et al. 2019). Theranostic agents such as magnetic nanoparticles (MNPs) induce redox cycling and catalytic chemistry, which causes the evolution of reactive oxygen species (ROS), leading to oxidative stress (Borm et al. 2006). ROS induces MMP activity in the nervous system leading to increased blood–brain barrier permeability and neuronal damage (Liu et al. 2007). Application of the nanotheranostics is safe, but an imbalance in the homeostasis gives toxic implications to many organs. The individual ions either iron, gold, or graphene may produce excessive free radicals (Madhyastha et al. 2019) in the brain and could be associated with multiple neurodegenerative disorders, including multiple sclerosis and Alzheimer's and Parkinson's diseases (Doraiswamy and Finefrock 2004). Toxicity of the nanotheranostics showed a large effect from cytotoxicity in vitro to transient and acute toxicity to unremarkable changes in vivo (Ma et al. 2008). The most toxic effect showed by MNP is accumulation in tissues, but with unremarkable histological changes in vital organs, concluding safety of the respective formulations. Ultrasound (US) theranostic-based biomedical technology is used frequently in the clinics as HIFU and SDT method. In both technologies, knowledge of material chemistry plays a key factor. In this technology core-to-shell design by solid to gas (perfluorocarbon) phase, interaction is an important factor to be considered. However, biosafety consideration is yet to evolve in this field. Platinum-conjugated drugs like, cisplatin, carboplatin, and oxaliplatin are used extensively in cancer biology. However, these drugs cause extensive damage to the liver and kidney. In recent years, development of Pt-based nanodrugs has seen progress. A major limitation of this nanodrug is toxicity in off-target organ and delay in rapid clearance by the cells of the reticuloendothelial system and mononuclear phagocyte system. Use of graphene in cancer theranostic treatment is still under development but rapidly growing because of its promising results

10.8 Conclusion

Considering the above importance of NPs in both diagnosis and therapeutics, it can be concluded that this is an advanced branch of nanotechnology with inimitable characteristics such as highly specific, targeted, blood–brain barrier crossing, drug delivery, imaging platform, unique transfection, labeling, bioseparation, as well as analytical and tissue engineering approaches. Yet, some challenges exist, such as nanotoxicity, environmental hazards, and target mismatching, which need to be

explored in the nearest future. Theranostic NP technology has drawn considerable interest in the cancer field, but application potential is hampered due to its toxicity property. Optical and smart design strategy to control the size and shape is another challenge in the application. Size of a particle plays an important role in the potential activity in vivo. Acceptable and adaptable size of a nanotheranostic drug for cancer cure is of 20 nm, but this size is not suitable or ideal for diagnosis due to poor imaging quality. Importantly posttreatment clearance from the cell is a major area for future research as the nanoresiduals may be a potential threat to cell architecture. More insight and research thoughts are also required on the concept of NP recycling from the cell as a novel challenge in biomedical research.

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