Nanotechnology in the Life Sciences

Devarajan Thangadurai
Jeyabalan Sangeetha
Ram Prasad *Editors*

Functional Bionano-materials

From Biomolecules to Nanoparticles

Springer

Nanotechnology in the Life Sciences

Series Editor

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Nano and biotechnology are two of the 21st century's most promising technologies. Nanotechnology is demarcated as the design, development, and application of materials and devices whose least functional make up is on a nanometer scale (1 to 100 nm). Meanwhile, biotechnology deals with metabolic and other physiological developments of biological subjects including microorganisms. These microbial processes have opened up new opportunities to explore novel applications, for example, the biosynthesis of metal nanomaterials, with the implication that these two technologies (i.e., thus nanobiotechnology) can play a vital role in developing and executing many valuable tools in the study of life. Nanotechnology is very diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale, to investigating whether we can directly control matters on/in the atomic scale level. This idea entails its application to diverse fields of science such as plant biology, organic chemistry, agriculture, the food industry, and more.

Nanobiotechnology offers a wide range of uses in medicine, agriculture, and the environment. Many diseases that do not have cures today may be cured by nanotechnology in the future. Use of nanotechnology in medical therapeutics needs adequate evaluation of its risk and safety factors. Scientists who are against the use of nanotechnology also agree that advancement in nanotechnology should continue because this field promises great benefits, but testing should be carried out to ensure its safety in people. It is possible that nanomedicine in the future will play a crucial role in the treatment of human and plant diseases, and also in the enhancement of normal human physiology and plant systems, respectively. If everything proceeds as expected, nanobiotechnology will, one day, become an inevitable part of our everyday life and will help save many lives.

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ISSN 2523-8027 ISSN 2523-8035 (electronic)
Nanotechnology in the Life Sciences
ISBN 978-3-030-41463-4 ISBN 978-3-030-41464-1 (eBook)
https://doi.org/10.1007/978-3-030-41464-1

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Nanotechnology is the most significant field of research today. It is a multidisciplinary subject encompassing various branches of science and technology. However the technological expertise is considered to be properly exploited when it is worthy to use for the benefit of mankind. In the journey of living a healthy life or saving the lives of people, nanotechnology is becoming a crucial driving force behind innovation in medicine and healthcare, with a range of advances including nanoscale therapeutics, biosensors, implantable devices, efficient drug delivery systems, regenerative medicine, and specific imaging technologies. Not only that, it has much potential for gene therapy, tissue engineering, and cell repair via nanorobots. Nanoscience has given us nanoparticles, nanomaterials, nanocomposites, quantum dots, and carbon nanotubes, and the process of manipulating them is conferred by nanotechnology. It is among the fastest growing areas of scientific research and is expected to have a substantial impact on healthcare.

The applications of nanotechnology under the medical health scope have had a major influence on how diseases are diagnosed and treated. While many developments have been primed, continuous investigation is being performed in this field. There are high expectations that in the coming few decades we will be able to combat against fatal diseases by much improved techniques and tools with the boon of nanotechnology. Nanotechnology is spreading its factions to address the key problems in the capacity of nanomedicine and healthcare by improving the prevention and monitoring of diseases. To bring a revolutionary breakthrough in the world of medicine or for personalized medicine, it needs many investigations, both theoretical and experimental, before coming to reality. Accurate and early detection of infectious disease remains one of the primary challenges of modern medicine. As with any advancement in diagnostics, the ultimate goal is to enable physicians to identify an infection as early as possible. Nanotechnology is expected to make diagnosis possible at the cellular and even the subcellular level with enhanced imaging techniques and high-performance sensors. Current achievements particularly with nanosensor platforms reveal high potential of fluorescent nanosensors that requires continuous in vivo monitoring of important biomarkers for clinical applications. These platforms hold great aptitude as alternatives to conventional recognition

elements, both for diagnostics and treatment purposes to improve healthcare. Quantum dots have to finally step up from pure demonstration experiments to real applications in imaging. These semiconducting nanocrystals are proving extremely beneficial for medical applications, such as high-resolution cellular imaging and enabling researchers to study cell processes at the level of a single molecule.

Advancements in nanotechnology applications highlight empirical research and emergent trends in scientific innovations in medication and healthcare. More lives could be saved by early diagnosis of cancer than by any form of treatment at progressive stages. Nanotechnology has the potential to take on the challenge of treating diseases like cancer by demonstrating a carbon nanotube chip and nanosilicon platform that capture and analyze circulating tumor cells in blood. Nanomedicine is relatively innovative ground for science and technology. By interacting with nanoscale biological molecules, nanotechnology opens up a vast field of application and research. The interactions between nanoscale devices and biomolecules can be conceived both in the extracellular medium and inside the cells of the human body. The nanometric scale makes it possible to exploit physical properties different from those observed at the microscopic scale. The diagnostic applications studied are applicable both for in vitro and in vivo diagnostics. A second area of nanomedicine with strong development is where synthetic nanoparticles are designed for the vectorization and delivery of pharmaceutically active ingredients. The use of these vectors improves the bio-distribution of drugs, focuses their targeting on pathological tissues with greater precision, and protects healthy tissues. The active ingredients can also be formulated so that a drug better permeates cell membranes, reducing the required dose. A third area of application is that of regenerative medicine, where nanotechnologies make it possible to design biocompatible materials intended to support the growth of cells used in cell therapy. Nanomedicine can potentially contribute to the development of personalized medicine, where a personal diagnosis would prescribe an effective personalized therapy. Nanorobots could actually be automated to repair particular diseased cells, functioning in a similar way to antibodies in the normal healing processes.

In the current status of nanomedicine, some potential applications are catching the attention of the present-day researchers and scientists. Nanopolymer scaffolds mimicking the natural pattern can substitute teeth or bone; supersensitive sensors can detect disease-causing biomolecules in a short time and from an extremely low concentration fluid; nano-tweezers can take images and manipulate nanosized objects inside the body during surgery. Nanotechnology is proving its excellence in all sectors of medicine, and hence the topic will be relevant at least for the coming two decades.

In this book, we highlighted the various applications of functional nanostructures for the advancement of medicine and healthcare tools. The book starts by describing how a wide variety of nanostructures is available for biomedical research and applications in Chap. 1. The importance of bionanomaterials for therapeutic purposes is discussed in Chaps. 2–5, wherein experts explain nanotechnology as a potential tool in exploring the herbal benefits and phytonanotechnology as a source to enhance wound-healing activity. Next, there is focus on the applications of nanomaterials in

drug delivery systems, stem cell research, tissue engineering, early diagnosis of cancer and its treatment in Chaps. 6–12. Broad examples of current developments in biosynthesis of silver nanoparticles, nano-antibiotics, and nanaprobiotics are elaborately described along with their applications in Chaps. 13–17. In the last two chapters of the book, the authors review the future perspectives and challenges for nanomedicines, biological risk assessment of advanced nanomaterials and their impact in the clinical scenario.

This book provides an in-depth investigation of therapeutic uses of nanoparticles, nanocomposites, nanofibers, and nanotubes in sensor application. Moreover, it encompasses the recent advancements in medicine and healthcare for revolutionizing the treatments for various fatal diseases and presents a number of major issues related to the progress of medical science through the knowledge of nanoscience and nanotechnology. Considering the impact of nanotechnology on regenerative medicines, treatment of diseases, and drug delivery systems, this book serves as a vital reference source for researchers and professionals in bioengineering, biotechnology, medicine, healthcare, pharmacy, and in other key areas of life sciences.

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Chapter 1
Nanoscience: Convergence with Biomedical and Biological Applications

Vikram Dalal and Sagarika Biswas

Abstract Nanoparticles (NPs) are tiny materials exhibiting a size range of 1–100 nm. Nanoparticles are used in biomedical applications like bioimaging, therapy, drug delivery, and biosensors; therapeutic applications such as treatment of cancer, inflammatory diseases, like inflammatory bowel diseases, inflammatory lung diseases, ophthalmic inflammatory diseases, and rheumatoid arthritis, wound healing, and cardiovascular diseases; nanoremediation; food industry; and agriculture. Metal oxides NPs and gold nanoparticles (AuNPs) are used in magnetic resonance imaging (MRI), positron-emitting tomography (PET), and computed tomography (CT). Nano-conjugates of drugs possess more cytotoxicity as compared to without NPs drug treatment of myeloid leukemia. Nanoparticles like gold half shell multifunctional NPs, polylactic-co-glycolic acid (PLGA) NPs, and solid lipid nanoparticles have been used for the delivery of drugs at the inflammatory site for the treatment of inflammatory diseases. AuNPs can be used as antioxidant, antimicrobial, and anti-inflammation agents for wound healing and burns. Nanoscale metals, carbon nanotubes, zeolites, fibers, metal oxides, and titanium oxides have been explored for remediation of toxic compounds. Nanoscale food additives can be used to increase the shelf life, flavor, texture, nutrient composition or even to detect the contaminants or pathogens in food. Biodegradable plastics such as polybutylene succinate (PBS), polylactic acid (PLA), and polyhydroxybutyrate (PHB) are polymers that can be easily degraded to nontoxic compounds in the presence of microorganisms. Nano-TiO_2 enhances the absorption of inorganic nutrients, accelerates the breakdown of organic substance, and can quench oxygen free radicals produced during the photosynthetic, so it can increase the rate of photosynthesis. Nanosensors like parathion, methyl parathion, pirimicarb, fenitrothion, and paraoxon are used to detect the residues of pesticides. Nanoparticles can also be used as biomarkers for the detection of viral, bacterial, and fungal pathogens in agriculture.

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_1

Keywords Cancer · Inflammatory disease · Nanochips · Nanoparticles · Nanopesticides · Nanosensors · Rheumatoid arthritis

1.1 Introduction

Nanotechnology is the field which deals with atomic, molecular, and supramolecular levels of molecules (1–100 nm) to understand the properties which can be utilized for human welfare (Roco et al. 2000). All man-made and biological systems have nanoscale organization level. The aim of nanotechnology is to determine the capability of the assembly of small molecules into large molecules and disassembly of large into small atomic substances. The matter at the nanoscale includes several non-covalent molecular interactions like electrostatic dipoles, van der Waal forces, hydrophobic interactions, fluidics and surface forces, and hydrogen bonds which play a vital role in reversible structural changes. Nanotechnology is classified into two categories such as wet and dry nanotechnology (Smalley 2002). Nanotechnology utilizes the nanoscale principles and techniques to understand the biosystems. Nanotechnology is converged with modern biology and medicine to produce more nanoscale materials which can be utilized for biological systems.

Metallic and metallic oxide nanoparticles (NPs) exhibit a wide range of application in areas of bioimaging, drug delivery, and treatment of diseases (Thurn et al. 2007; Arvizo et al. 2012; Doane and Burda 2012; Sasidharan and Monteiro-Riviere 2015). Nanoparticles are used in several therapeutic applications like the treatment of infectious and inflammatory diseases and constructions of membranes, mats, and hydrogels (Gaharwar et al. 2014; Ikoba et al. 2015; Zhan et al. 2015). They have remarkable properties like optical sensitivity, conductivity, and reactivity due to their unique physicochemical properties like controllable shape and size, high surface area-to-volume ratio, and superparamagnetism (Alam et al. 2009; Ramesh et al. 2014).

Nanotechnology investigative methods are used to determine the cellular processes, biological processes, and systems biology. It also plays a major role for measurements at the atomic and subcellular levels to understand the cellular processes like self-replicating, self-repairing, and information-rich molecular machines (Ishijima and Yanagida 2001; Müller et al. 2002). They are also used for shed light on molecular mechanistic properties and dynamics both in vitro and in vivo which directly investigate enzyme reactions, DNA transcription, molecular motors, and cell signaling. Atomic force microscopy (AFM) can be used to measure the intermolecular mechanics of single protein molecule (Ikai et al. 2002). Nanotechnology can also be used to measure the small RNAs and their gene expression in vivo

(Couzin 2002). Biological force microscope can quantitatively measure adhesion and interfacial forces between minerals or biological systems to living bacteria (Lower et al. 2001). Nanotechnology is used to visualize structural changes of protein in complex living systems by variation in visible color changes (Baneyx et al. 2001). It can also be used to find the Brownian motion which can use to explain kinesin motion along microtubules (Fox and Choi 2001).

Nanotechnology is used in medical field to define the manipulation of matter on the atomic or molecular scale. Several materials, like silver, gold, silica, cerium, iron, carbon, zinc, copper, nickel, titanium dioxide, and magnesium, are used in the fabrication of NPs. Gold is highly preferred in the construction of NPs due to its hydrophobicity. The first NP was produced from gold and gold-iron oxide magnetic glycol nanoparticles (De la Fuente et al. 2006). The interactions of ligands with atoms on the surface of particle play a major role in the determination of physiochemical properties of NPs. Extensive efforts are made to produce the NPs smaller than 6 nm so that they can pass through the kidney. Globular gold nanoparticle (AuNP) with a hydrodynamic diameter (HD) < 6 nm can easily cross the glomerular capillary wall, whereas AuNP HD > 8 nm cannot cross it. The surface charge on NPs also plays an important role in kidney filtration like positively charged NPs with HD (6–8 nm) can easily cross the kidney capillary wall while negatively charged NPs cannot cross it.

Application of nanotechnology includes the drug delivery in vivo and in vitro and diagnostics of the production of biocompatible materials and nutraceuticals (Duncan 2003; de Jong et al. 2005; Ferrari 2005). It has been reported that NPs that have a size $\leq$100 nm can be used for medical purposes (Rogers-Hayden and Pidgeon 2007). Nanoparticles are utilized in medical applications due to their unique properties like quantum properties, surface-to-mass ratio that is much larger than that of other particles, and adsorption capacity to carry other compounds like probes, proteins, and drugs. In drug delivery, NPs size should be less than 0.1 μm or 100 nm. The composition of NPs can be varied like source materials may be biological lipids, dextran, lactic acid, phospholipids, chitosan, or chemicals like silica, carbon, metals, and various polymers. The interactions of biological components of NPs are different as compared to nonbiological components. Biodegradable NPs are required in drug delivery to transport and release the drug at the site of infection.

Magnetic nanoparticles (MNPs) are highly used in diagnostic and therapeutic approaches. Magnetic nanoparticles are used in magnetic resonance imaging (MRI) and carriers of targeted drug delivery (Pankhurst et al. 2003; Corot et al. 2006; Dobson 2006). They have a wide range of applications in diagnosis, detection, and treatment of diseases like cancer, neurological disorders, and cardiovascular diseases (Corot et al. 2004; Ferrari 2005; Wickline et al. 2007). MNPs have been used for delivery of pharmaceuticals through magnetic drug targeting (MDT) (Senyei et al. 1978).

1.2 Diagnostics

1.2.1 MRI

Iron-based NPs can alter the magnetic field while AuNPs are non-paramagnetic and they are biological compatible but cannot be used for contrast of blood or tissues. Magnetic resonance imaging (MRI) contrast agents should have the ability to alter the proton relaxation and can perturb the local magnetic field around the proton. The perturbed field of superparamagnetic material in MRI can influence water protons in several cell layers around its location (Shilo et al. 2012). The first generation of magnetic nanoparticles (MNPs) is already in clinical use for MRI, while the second generation of MNP which possess a longer blood half-life will be approved in the future. Gold nanoparticles are comprised of faradaic properties, but they are not used in MRI due to the absence of nonmagnetic properties. It has been reported that magnetic properties exhibiting AuNPs can be used in MRI imaging (Mohs and Provenzale 2010). Superparamagnetic MNP is comprised of iron-oxide surrounded with polymer coat or carbohydrate, which plays a vital role in cardiovascular molecular imaging. The physical properties, size, and pharmacokinetics of MNPs are important factors for using them as molecular imaging of myocardial injury and atherosclerotic plaques (Xing et al. 2014).

1.2.2 Positron-Emitting Tomography (PET)

Radiolabeled positron-emitting isotopic NPs have been developed to determine the tumor enzyme activities and tumor receptor levels (Pérez-Campaña et al. 2013). Metal oxide NPs have been used for the production of PET imaging probes. It has been reported that these probes can activate $_{18}$O-enriched aluminum oxide (Al_2O_3) NPs to produce 18F-labeled NPs (Pérez-Campaña et al. 2013).

1.2.3 Ultrasound

Solid NPs can be used for ultrasonic grayscale images in mouse liver and tissue phantoms in vivo. Agarose (1–2.5%) mass concentration along with silica nanospheres (100 nm) can be utilized for high-resolution ultrasound imaging (Liu et al. 2006). It has been found that platinum nanoparticles (Pt-NPs) in combination with 1 MHz ultrasound at an intensity of 0.4 W and 10% duty factor can be used to determine the cell cycle analysis and DNA fragment assay.

1.2.4 Computed Tomography

Particular regions in the human body can be enhanced for the diagnosis of a range of disease conditions. Computed tomography (CT) imaging needs millimolar contrast agent to trigger enough contrast in the desired organ, so much larger amount of contrast is required. Nanoparticle can amplify the contrast which decreases the exposure of the body to harmful CT contrast agents. High atomic number materials such as bismuth and gold are preferred due to the production of high contrast and less radiation exposure of patients as compared to iodine-based contrast agents. It has been reported that mm-sized human breast tumors in mice can be visualized 1.6 times more efficiently by using AuNPs as compared to passive targeting agents (Hainfeld et al. 2004). It was found that the uptake of AuNPs was 22-fold higher in tumor periphery as compared to surrounding muscles.

1.3 Therapy and Drug Delivery

The whole system (10–100 nm) of drug delivery with NPs comprises two components one of which is pharmaceutically active ingredients (Baran and Hasirci 2002; Cascone et al. 2002; Duncan 2003; Ferrari 2005). The complete system consists of a special function related to treatment, prevention, and diagnosis of diseases which is sometimes called smart drugs or theragnostics (Lavan et al. 2003). The main aims of nanobiotechnology in drug delivery are:

- Specification of drug delivery
- The decline in toxicity along with the maintenance of therapeutic effects
- Safety and biocompatibility
- Enhance the production of new face drugs or medicines

The basic knowledge required before the selection of appropriate carriers as a drug delivery system is: (a) incorporation and release of the drug, (b) stability and shelf life of drug and carrier, (c) biocompatibility, (d) biodistribution of drugs and carriers, and (e) mode of action of the drug.

1.3.1 Cancer

Cancer is due to the spreading of tumor cells which are resistant to killing by the conventional drugs. Researchers developed the NPs which target the specific tumor cells. It has been found that NPs can deliver arginine-glycine-aspartate (RGD) peptide at the tumor site (Poon et al. 2015). Gold nanoparticles (AuNPs) were used as

a multimodality imaging agent for brain glioma (Lai et al. 2015). Chemotherapy and radiation therapy along with surgery are the therapeutic strategies for the treatment of cancer. High-intensity ionizing radiations have to reach with high accuracy to the tumor tissue to cause death of these cells. But radiation therapy can cause injury to the surrounding cells also. Another disadvantage of radiation therapy is some tumor cells receive a lower intensity of radiations as they are farther away from the site of radiation in the body. Moreover, tumor cells can develop resistance for radiation, so they cannot be affected by radiations. The three major approaches adapted for the improvement of radiation therapy are the following: (1) enhancement of radiosensitization of tumor tissues, (2) reversing of radiation resistance in tumor tissue, and (3) enhancing radioresistance of healthy tissues (Kwatra et al. 2013). The main aim of radiotherapy is to enhance the efficiency of tumor killing with a reduction in healthy tissue damage. The enhancement of AuNP radio becomes an investigative area to increase the efficiency of ionization radiation in a biological system. There is still a lot of work required to improve the targeting of AuNPs at the tumor site. The biodistribution of AuNP was measured at the cellular level (McQuaid et al. 2016).

The role of kilovoltage radiosurgery with AuNPs for AMD (age-related macular degeneration) has been found (Brivio et al. 2015). It has been reported that the delivery of a dose of X-ray radiation treated with AuNPs is reduced to half as compared to treated without AuNPs X-ray (Brivio et al. 2015). It has been found that AuNPs can decrease the growth of colorectal cancer cells after treatment with cold plasma (Irani et al. 2015). Cheng et al. (2014) determined that NPs play a synergistic role with cold plasma (Cheng et al. 2014). Nanoparticles' addition results in the decline of cell viability which makes cold plasma more efficient. Nanoparticles play a major role in radiotherapy, proton therapy, and cold plasma therapy. Nano-conjugates of sorafenib, lestaurtinib, lestaurtinib, and quizartinib possess more cytotoxicity as compared to normal sorafenib, lestaurtinib, lestaurtinib, and quizartinib for the treatment of myeloid leukemia (Simon et al. 2015). It is determined that drugs for cancer are not developed due to poor stability and poor solubility (Haynes et al. 2016). It is reported that the attachment of AuNPs to drugs enhances the efficiency of drug delivery and triggers the mitochondrial cell death. Tumor angiogenesis-targeted radiosensitization therapy used the ionizing radiation which can be induced by cyclic RGDyC peptide conjugated with AuNPs (Yang et al. 2016). AuNP arginine-glycine-aspartate (RGD) probes are utilized as radiosensitizers to enhance the radiotherapy of tumor cells. Several cancer therapies used the combination of CT with radiotherapy. Yang et al. (2016) used the optimal size of NPs to enhance the CT imaging and radiotherapy. They trapped the larger particles in the stroma around the tumor by enhanced permeability and retention (EPR) effect. The sizes of 3–50 nm trapped particles were investigated and confirmed that 13 nm particles were optimum for dual application. Shilo et al. (2015) found the AuNPs can be used with radiosensitizers for neck and head cancer.

1.3.2 Inflammatory Diseases

Local delivery of anti-inflammatory drugs is necessary to avoid the side effects of the drug. Several nanoparticles like polylactic-co-glycolic acid (PLGA) nanoparticles, gold half shell multifunctional nanoparticles, and solid lipid nanoparticles (SLN) have been used to increase drug localization at the target site (Gref et al. 1994; Ye et al. 2008; Lee et al. 2012b). Nanoparticles like AuNPs, PLGA, actarit-loaded solid lipid nanoparticles, and gold half shell nanoparticles have been used for the treatment of inflammatory diseases. Gold nanoparticles exhibit various properties like facile surface modification, biocompatibility, and versatile conjugation, so they can be utilized for various biomedical applications (Ghosh et al. 2008; Giljohann et al. 2010; Thakor et al. 2011; Lee et al. 2012a). Gold nanoparticles have an antiangiogenic property which can bind to a vascular endothelial factor which plays a pivotal role in the pathogenesis of rheumatoid arthritis (RA) (Firestein 2003; Bhattacharya et al. 2004; Tsai et al. 2007).

Nanoparticles targeted to macrophages can be used for the treatment of autoimmune blood disorders like rheumatoid arthritis and diabetes (Barrera et al. 2000; Chellat et al. 2005; Moghimi et al. 2005). Intravenously injected nanoparticles have the ability to accumulate in the organs like the liver and spleen, so they can be utilized for the treatment of RA to remove the particulates from the blood (Moghimi et al. 2001). The spleen produces the macrophages which further cause the inflammatory responses. The spleen can be targeted to enhance the therapeutic efficacy and to decline the side effects of diseases modifying antirheumatic drugs (DMARDs). In mice, it has been found that actarit plays an important role in the inhibitory effect of progression lesions (Yoshida 1987). Actarit can improve the plasma albumin/globulin ratio of adjuvant RA rats (Fujisawa et al. 1990, 1994). However, oral administration of actarit leads to a higher accumulation of actarit in the gastrointestinal tract and kidney as compared to spleen (Sugihara et al. 1990). Most adverse effects of actarit are related to gastrointestinal disorders (Matsubara 1999). DMARDs can induce the renal dysfunction and urinary abnormalities in RA (Makino et al. 2002). Biodegradation of polymers into glycolic acid, metabolite monomers, and lactic acid can be obtained by using PLGA. It can be easily metabolized in Krebs cycle, hence highly used for drug delivery and biomaterial applications (Kumari et al. 2010). Polylactic-co-glycolic acid NPs are internalized in the body by clathrin-mediated endocytosis and pinocytosis.

1.3.2.1 Inflammatory Bowel Diseases

Crohn's diseases and ulcerative colitis possessed different pathogenesis, but both required corticosteroids and 5-aminosalicylic acid to trigger the remission of the treatment. However, the significant increment of uptake is required to equilibrate the pharmacological effects, but it can result in adverse side effects (Meissner and

Lamprecht 2008). Polylactic-co-glycolic acid-based NPs are a promising candidate for the delivery of drugs to the colon in inflammatory bowel diseases (IBD). Smaller NPs can be taken up easily in the areas of inflammations (Lamprecht et al. 2001a). High negative charged PLGA can be used for drug delivery in ulcerated tissues. Confocal laser endomicroscopy showed that PLGA NPs accumulate in ulcerous lesions of inflammatory bowel disease patients, and further nanoparticles lead to the release of anti-inflammatory drugs (Schmidt et al. 2010). Polylactic-co-glycolic acid NPs can accumulate in the inflamed regions and can release the entrapped drugs in the inflammation regions (Lamprecht et al. 2001b). Tacrolimus-engulfed PLGA NPs can release the tacrolimus in the inflamed regions, and it protects the tacrolimus from P-gp efflux and mucosal metabolism (Lamprecht et al. 2005).

1.3.2.2 Inflammatory Lung Diseases

Polylactic-co-glycolic acid encapsulated PS-341 is a chymotryptic threonine protease inhibitor that can decrease the inflammation of cystic fibrosis. *Pseudomonas aeruginosa*-induced inflammation can be controlled by PLGA encapsulated PS-341, while the direct administration of PS-341 did not show any effect on inflammatory neutrophils and macrophages (Vij et al. 2010). Oral administration of PLGA NPs encapsulating curcumin increases the treatment in cystic fibrosis mice as compared to direct delivery of curcumin (Cartiera et al. 2009).

1.3.2.3 Ophthalmic Inflammatory Diseases

Direct administration of free drugs in the eyes is not preferable due to poor bioavailability in tissues, impermeability of drugs, and rapid turnover in the corneal epithelial membrane. So, only 5% of free drugs reach up to the intraocular tissues after cornea penetration (Zhang et al. 2004). Polylactic-co-glycolic acid NPs have a suitable size for drug delivery in ophthalmic inflammatory diseases. Polylactic-co-glycolic acid and its metabolites are nontoxic and biodegradable (Garinot et al. 2007). Nonsteroidal anti-inflammatory drugs are used to cure seasonal allergic conjunctivitis, postoperative inflammation, and reduction of bacterial colonization and inhibit bacterial adhesion to human corneal cells (Schalnus 2003). Polylactic-co-glycolic acid encapsulated with poloxamer 188 (P188) can be used to graft cationic polymer on the anionic surface of NPs. The NPs reveal a better anti-inflammatory efficacy and no toxicity as compared to eye drops. It has been found that PLGA encapsulated flurbiprofen shows high precorneal residence time at the inflammatory site (Vega et al. 2008; Araújo et al. 2009). Polylactic-co-glycolic acid can be used in combination with other nanoparticles to modify NP properties to target it to the more specific site in the eye. PLGA/Eudragit at RL (75:25) encapsulated with ciprofloxacin reveals a higher drug concentration in the tear film (Dillen et al. 2006).

1.3.2.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic disorder which can cause joint and bone destruction. RA involved environmental factors, genetic risk factors, and activation of immune responses. RA is characterized by inflammation such as heat, redness, pain, swelling, and loss of function of connection (Dalal et al. 2017). RA can be diagnosed by clinical parameter, the presence of anti-cyclic citrullinated peptide (CCP)/rheumatoid factor/anti-mannose binding lectin (MBL), c-reactive protein, radiographic evidence of joint destruction, and complete blood count. Several anti-inflammatory drugs like glucocorticoids are used for the treatment of RA, but they can cause serious systemic side effects (Lee and Kavanaugh 2003). It has been found that macrophage can phagocytose the PLGA nanoparticles but PLGA microsphere stayed in the synovium of rat (Horisawa et al. 2002b). Betamethasone sodium phosphate (BSP), a steroid encapsulated in PLGA, has been used to release the drug in animal arthritis model (Higaki et al. 2005). Direct intra-articular injection of BSP encapsulated with PLGA nanoparticles enhances the pharmacological efficacy (Horisawa et al. 2002a).

Monocyte-derived myeloid cells comprise potential effectors and scavenger cells during inflammatory processes (Taylor et al. 2003; Jongstra-Bilen et al. 2006; Iwamoto et al. 2007). Uncontrolled activation of myeloid cells can result in chronic inflammations. Myeloid cell-mediated chronic inflammation plays a crucial role in chronic illnesses like cardiovascular, neurodegenerative, autoimmune, and pulmonary metabolic disorders (Boyd et al. 2008; Croce et al. 2009; Deng et al. 2009; Qu et al. 2009; Soehnlein and Weber 2009). *Curcuma longa* (turmeric) has antineoplastic, anti-inflammatory, chemopreventive, and antioxidant properties (Dhillon et al. 2006; Anand et al. 2008; Ravindran et al. 2009). Thus, curcumin has been encapsulated in biodegradable microspheres, liposomes, hydrogels, and biodegradable nanoparticles, and cyclodextrin and exosomes can deliver the curcumin to activate the myeloid cells (Choi et al. 2009; Narayanan et al. 2009; Sun et al. 2010). This technology can be used to treat inflammatory cells by releasing the curcumin at the site of inflammation.

Tumor necrosis factor-α (TNF-α) secretion from macrophages plays an important role in the progression and development of rheumatoid arthritis. Double-stranded RNAs cleaved into very short 21–22 mer fragments which can enter into a ribonuclear protein complex called RNA-induced silencing complex. Small interfering ribonucleic acid (siRNA) antisense strand can guide the RNA-induced silence complex for the degradation of specific mRNA (Elbashir et al. 2001). It has been found that direct articular injection of TNF-α-specific siRNA can decrease the joint inflammation in murine collagen-induced arthritis (CIA) (Schiffelers et al. 2005). In mice, TNF-α-specific siRNA encapsulated with cationic lipid-based nanoparticles shows anti-inflammatory effects (Khoury et al. 2006). Knockdown of TNF-α chitosan/siRNA nanoparticles can control the inflammation in RA. Chitosan nanoparticles-engulfed anti-TNF-α dicer substrate (DsiRNA) can result in the

knockdown of TNF-α in primary peritoneal macrophages (Howard et al. 2009). It has been reported that nanoparticle-mediated TNF-α knockdown in peritoneal macrophage can be used to decline the local and systemic inflammation (Howard et al. 2009).

Gold nanoparticles (AuNPs) can inhibit the receptor activator of nuclear factor-κB ligand (RANKL)-induced synthesis of osteoclasts by quenching reactive oxygen species (ROS) which can cause the destruction of cartilage and bones (Sul et al. 2010). AuNPs can be utilized as a nanoprobe for the diagnosis and detection of RA. Tocilizumab (TCZ) humanized antibody against interleukin-6 (IL-6) can be used to inhibit the pathogenesis of RA. It has been observed that hyaluronate-gold nanoparticle/tocilizumab (HA-AuNP/TCZ) complex can be used for the treatment of RA (Lee et al. 2014). Lipophilic anti-RA drug is incorporated into solid nanoparticles. Solid lipid nanoparticle (SLN)-encapsulated actarit can be used as a passive targeting agent in RA and may reduce dosing frequency, doses, and toxicity and improve compliance (Ye et al. 2008). Methotrexate (MTX) is a disease-modifying antirheumatic drug (DMARDs) which is used for the treatment of RA (Saag et al. 2008). MTX exhibits the good therapeutic efficacy, but the long-term use of MTX can cause a serious systemic infection like bone marrow suppression, infections, and hepatitis (Schnabel and Gross 1994; Van Ede et al. 1998). Increase in dose concentration of MTX in RA patients can cause several disorders in bone marrow dysfunction and intestinal lung diseases. Alternatively, for better treatment, methotrexate containing Arg-Gly-Asp (RGD)-attached gold (Au) half shell nanoparticles can be used for the delivery of MTX at inflammation site which further can be used for the treatment of rheumatoid arthritis (Lee et al. 2012b).

Nonsteroidal anti-inflammatory drugs (NSAIDs) can inhibit cyclooxygenase which converts arachidonic acid into prostaglandins, so NSAIDs can be utilized for the treatment of inflammatory disorders. NSAIDs decrease the anti-inflammatory and antiproliferative which further can suppress the expression of NF-kB (Kusunoki et al. 2008). siRNA and glucocorticoids can be co-delivered by encapsulating into PLGA to suppress the expression of unnecessary proteins and genes of RA (Park et al. 2012).

1.3.3 Wound Healing and Burns

This area considered the healing of wounds and burns without the formation of a scar. Synthesized AuNPs from *Coleus forskohlii* have been used to cross-check the closure of wounds in rats (Naraginti et al. 2016). Gold nanoparticles promote closure of the wound and decrease the excision of wounds. Gold nanoparticles loaded to electro-spun silk to check the anti-inflammatory action of AuNPs (Ju et al. 2016). Wound healing models are used to determine the action of matrices which

incorporate AuNPs. It decreases the scar tissue and increases matrix properties and good wound closure properties. Methylcellulose gel with AuNPs was used in wound healing of surface burns (Volkova et al. 2016). Fluorescent agents were labeled with NPs to determine the wound healing process (Pittet et al. 2006). So AuNPs can be used as antioxidant, antimicrobial, and anti-inflammation agents for wound healing and burns.

1.3.4 Cardiovascular Disease

Restenosis is a common adverse event in cardiovascular disease. Nanoparticles conjugated with epigallocatechin-3-gallate could be used as a therapeutic approach to drug-coated stents to prevent restenosis (Khoobchandani et al. 2016). Laminin and avid receptors can internalize the AuNP conjugates. This type of NPs can be a target to the specific parts of the cardiovascular system which can reduce the surgical intervention. Apolipoprotein AI (ApoAI) and ApoB are the risk indicators in cardiovascular diseases. Immune resonance scattering is used to measure the level of ApoAI and ApoB in serum (Jiang et al. 2006). Goat antihuman ApoAI and ApoB antibodies conjugated with 9 nm AuNPs were used in trisodium citrate method. The immune reaction between antigens and AuNP-labeled antibodies took place in NaH_2PO_4-Na_2HPO_4 buffer solution. Transmission electron microscope (TEM) was used to observe the shape of gold nanoparticles. They found this method showed good selectivity and high sensitivity for the quantitative determination of ApoAI and ApoB in human serum.

1.3.5 Neuroimmunology

The development of NPs used in the brain is limited due to the difficulty of passing of blood-brain barrier (BBB) by NPs. This barrier protects the brain from toxic and harmful agents, but it also stops the delivery of drugs to the brain. Several strategies have been used to deliver drugs across this barrier, but some of these drugs may structurally damage the BBB (Jain 2012). Nanobiotechnology-based drug delivery methods have been used to deliver the drugs to the brain. These strategies use nanoparticles conjugated with drugs for a therapeutic approach. Size, coating, and surface charge of NPs play a major role in the passage of NPs across the BBB (Shilo et al. 2015). The size of 20 nm AuNP is optimal for the maximal surface area to cross the BBB, while the size of 5 nm or less is required to pass out the kidney. Morris et al. (2016) studied the 15 nm diameter of AuNPs for the passage of fluid into and out of the brain (Morris et al. 2016).

1.3.6 Antibiotics

Most physiological measurement devices need direct contact with patients. Antimicrobial peptide conjugated with AuNPs was used to check antimicrobial activity against Gram-negative and Gram-positive as well as against multidrug resistance bacteria (Durán et al. 2011). Coatings of AuNPs were compatible with plasma and showed low toxicity for human cells. Moreover, these coatings can be easily transferred to glass, titanium, and other commonly used substrates in medical devices. Broad-spectrum bactericidal activity against Gram-negative and Gram-positive bacteria was checked by amoxicillin coated with AuNPs (Kalita et al. 2016). These conjugates also possess anti-multiresistant *Staphylococcus aureus* (MRSA) activity. Chitosan-streptomycin AuNP conjugates can be used to inhibit planktonic cell growth in Gram-negative bacteria (Mu et al. 2014).

1.3.7 Tissue Engineering

Tissue repair of wound and organ or limb is of considerable interest in the last decade. Biomaterial patches consisted of decellularized tissue conjugated with AuNPs can integrate with host tissue, shows its importance as vascular repair and blood-contacting (Moravej and Mantovani 2011). Cardiac tissue engineering-related NPs have potential therapeutic importance for end-stage heart failure and can give solution for heart donor problems. Polycaprolactone (PCL) scaffold-conjugated AuNPs can be used to increase the proliferation and differentiation of mesenchymal stem cells (MSCs) in cardiac tissues, and this strategy can be used to repair myocardial infarction (Sridhar et al. 2015). The 3D nanoscale nanoscaffold consists of polymer fibers used for adherence and rebuilding of neural tissues (Feng et al. 2015). Carbon nanotubes can promote differentiation and neuron growth, so they can be used for the treatment of central nervous system injuries by enhancing the axon regeneration and synaptogenesis (Bokara et al. 2013).

1.4 Other Applications

1.4.1 Nanoremediation

Nanoremediation utilizes the reactive nanomaterials for the detoxification or transformation of harmful or toxic compounds to nontoxic compounds. These materials can reduce chemical and catalyze the concerned pollutants (Prasad and Aranda 2018). Nanomaterials are very useful for in situ remediation due to their innovative surface and small size. Nanoscale metals, carbon nanotubes, zeolites, fibers, metal oxides, and titanium oxides have been explored for remediation. Nanoscale zero-valent iron (nZVI) size in the range of 10–100 nm in diameter is used in remediation. Nobel

metals such palladium, copper, and silver can be utilized as a catalyst in chemical reactions. The second metal results in catalytic synergy with Fe and enhances the mobility into the ground (Trantyek and Johnson 2006; Saleh et al. 2007; EPA 2008). These bimetallic nanoparticles (BNP) may consist of more than two different metals and second metal is used to enhance the rate of Fe oxidation or electron transfer (EPA 2008). Some noble metals like palladium can make remediation more efficient by catalyzing the hydrogenation and dechlorination (Zhang 2005; Zhang and Elliott 2006; EPA 2008). Zhang (2005) first reported the field application of nZVI. Nanoparticles are very reactive and remain active in water and soil up to 8 weeks and can easily pass with groundwater up to 20 nm. Random or Brownian movement dominates the physical movement or transport of NPs along with water due to the small size of NPs. The movement of microscale metals and micrometer-sized particles are governed by gravity-induced sedimentation due to the high density and size. Some NPs can flow easily with groundwater and can sustain in water for a long time, while others can filter out and bind to soil particles. These properties of NPs make them a useful in situ remediation which can hold back emanating plumes (Henn and Waddill 2006).

1.4.2 Food Industry

Food cultivated, produced, processed, or packaged by using nanotechnology is referred to as nanofood (Morris 2007). Nanotechnology includes food security, disease treatment delivery methods, new methods for pathogen detection, and new techniques for cellular and molecular biology. Nanotechnology is used for further advancements in the food industry as:

- Increment in the security of manufacturing, processing, and shipping of food products.
- Using sensors or probes for detection of contamination.
- Devices which can track the shipping products.
- Systems to integrate the sensing, localization, and remote control of food products.
- Increase security of processing and transportation of food.
- Encapsulation and delivery system which can carry, protect, and deliver food ingredients at a specific site.

Nanotechnology plays an important role in the food industry. Nanotechnology is used mainly in two approaches in nanofood applications: food additives (nano inside) and food packaging (nano outside). Nanoscale food additives can be used to increase the shelf life, flavor, texture, and nutrient composition or even to detect the contaminants or pathogens in food (Ravichandran 2010; Prasad et al. 2017a). The main aim of innovative packaging is to reduce the spoilage of food. Nanosensors can be used to increase the efficiency of food production and processing. Silver is an antimicrobial agent used to decline the bacterial growth so that food can be preserved for a long time. Silver nanoparticles (AgNPs) can reduce the growth of bacteria to 98% during a period of 24 h (Wesley et al. 2014).

Food packaging is one of the major applications of nanotechnology in the food sector. It is found that 400–500 products are packaged as nanopackaging for commercial use (Reynolds 2007). Nanopackaging can also be used to increase the shelf life of enzymes, antioxidants, nutraceuticals, antimicrobials, and flavors (Cha and Chinnan 2004). Several new nanotechnology approaches such as nanocomposites, carbon nanotubes, biosensors can be used for food packaging (Ishrat et al. 2018; Singh et al. 2020). Waxy coatings are generally used for the preservation of food such as cheese and apples. A 5 nm wide nanoscale edible coating has been developed on several food items such as cheese, vegetables, candies, chocolate, french fries, and bakery products by using nanotechnology (Morillon et al. 2002; Cagri et al. 2004; Rhim 2004).

Nanosensors can be utilized to detect the external or internal conditions of food products which can be used to determine the shelf life of products. Nanosensors can detect the level of humidity or temperature over time, and then it can suggest the relevant condition of the food product. Nanosensors in plastic packaging can easily detect the gases produced by food when it spoils, and the package itself changes color to alert the spoilage of food (Singh et al. 2020). It has been reported that nano magnesium oxide, nano silver, nano titanium dioxide, nano copper oxide, and carbon nanotubes can also be used in the future for antimicrobial food packaging (Doyle 2006; Chaudhry et al. 2008; Miller and Senjen 2008). Enzymes are also utilized to scavenge the oxygen in polyethylene packaging films (Lopez-Rubio et al. 2006).

1.4.3 Biodegradable Plastics

Biodegradable plastics are polymers which can be easily degraded to nontoxic compounds under the appropriate physical condition like temperature, moisture, and oxygen availability in the presence of microorganisms (Wesley et al. 2014). Biodegradable polymers can be categorized into the following classes on the basis of their sources: (a) directly extracted polymers from biomass (i.e., polypeptides, protein, polynucleotides, and polysaccharides), (b) polymers synthesized from renewable bio-based monomers such as bio-polyester or polylactic acid, and (c) polymers synthesized from microorganism or genetically modified microorganisms (bacterial cellulose, curdian, xanthan, pullan, polyhydroxybutyrate). The most studied biodegradable plastics are starch, and derivatives are polybutylene succinate (PBS), polylactic acid (PLA), and polyhydroxybutyrate (PHB).

1.4.4 Agriculture

The effects of nanomaterials on plant germination and growth have been studied to promote its use for agricultural applications (Prasad et al. 2017b, c, d). Zheng et al. (2005) determined the effects of nano-nano-TiO_2 on the germination and growth of spinach seeds (Zheng et al. 2005). It was found that the treatment of seeds with

nano-TiO_2 produced 73% more dry weight and three times higher photosynthetic rates in plants as compared to the control. The growth rate of spinach seeds was inversely proportional to the nanomaterials, which shows that the usage of small nanomaterials would increase the efficiency of germination. The main reason of enhancement in growth rate is due to the photo-generation and photo-sterilization of active oxygen species such as hydroxide anions and superoxide by nanomaterials which can increase the capacity of seed stress resistance and boost the capsule penetration for the uptake of oxygen and water for fast seed germination. It has been found that nano-TiO_2 can increase the absorption of inorganic nutrients, accelerate the breakdown of organic substance, and quench oxygen free radicals produced during the photosynthetic, so can increase the rate of photosynthesis.

Nano-pesticides can enhance the wettability and dispersion of agricultural formulations and the movement of unwanted pesticides (Bergeson 2010; Bhattacharyya et al. 2016). Biocomposites and nanomaterials possess useful properties like crystallinity, permeability, stiffness, solubility, thermal stability, and biodegradability required for the formulations of nano-pesticides (Bordes et al. 2009; Bouwmeester et al. 2009). Nano-pesticides exhibit a large surface area which can increase its efficiency for target (Yan et al. 2005). Some of the nano-pesticide delivery techniques such as nanocages, nanoencapsulation, nanoemulsions, and nanocontainers are used for the protection of plants (Bouwmeester et al. 2009; Lyons and Scrinis 2009; Bergeson 2010). It has been found that sodium dodecyl sulfate (SDS)-modified photocatalytic TiO_2/Ag nanomaterial conjugated with dimethomorph (DMM) can be used as nano-pesticide to enhance the agricultural production (Yan et al. 2005). Nanomaterials modified with SDS enhance the absorption of DMM. It has been reported that encapsulated nano-imidacloprid with the above properties can be used to control the pests during vegetable production in vegetables (Guan et al. 2010).

Nanosensors used for the detection of pesticide residue offer a low detection limit, fast responses, small sizes, high sensitivity, and super selectivity (Liu et al. 2008). Some of the nanosensors used for the detection of pesticide residues are parathion, methyl parathion, pirimicarb, fenitrothion, and paraoxon (Vamvakaki and Chaniotakis 2007; Kang et al. 2010; Parham and Rahbar 2010). Enzyme-based biosensors for organophosphate, organochlorine, and carbamate residue detection were studied (Van Dyk and Pletschke 2011). These biosensors use carbon, hybrid titanium (Ti), Au, Au-platinum, and lead dioxide (PbO_2) for the immobilization of enzymes on a sensor substrate which can enhance the sensitivity of the sensor.

In agriculture, nanoparticles can be used as biomarkers for the detection of viral, bacterial and fungal pathogens in agriculture (Boonham et al. 2008; Yao et al. 2009; Chartuprayoon et al. 2010). Nanoparticles can be used directly or used as a diagnostic tool for the detections of diseases or as indicators of compounds. Nanochips consist of fluorescent oligo capture probes which can be used to detect the hybridization (López et al. 2009). Nanochips have a high specificity and sensitivity for nucleotide changes of bacteria and viruses (López et al. 2009). Fluorescent silica NPs in combination with antibody were used for the detection of *Xanthomonas axonopodis* pv. *vesicatoria* which can cause bacterial spots disease in Solanaceae plants (Yao et al. 2009). Nano-gold-based immunosensor was developed which can

detect Karnal bunt (*Tilletia indica*) disease in wheat by using surface plasmon resonance (SPR) (Singh et al. 2010). Fungi (*Sclerotinia sclerotiorum*) can be detected by the modified gold electrode with copper nanoparticles (CuNPs) by measuring the levels of salicylic acid in oilseed (Wang et al. 2010).

1.5 Conclusion

Nanotechnology helps to understand the atomic, molecular, and supramolecular levels of molecules. Nanotechnology was used to find the cellular and biological processed. Metallic NPs exhibit a high surface area-to-volume ratio, optical sensitivity, and superparamagnetism. Nanoparticles are widely used in biomedical applications like drug delivery, biosensors, and bioimaging and therapeutic applications such as treatment of inflammatory diseases and construction of hydrogels, mats, and membranes. Nanoparticles produce the radicals which can lead to oxidative stress that can result in cell injury and cell death. NPs can interact with lipids, proteins, and DNA and degrade protein and membranes.

Magnetic nanoparticles are in clinical trial for usage in magnetic resonance imaging (MRI). Iron nanoparticles with magnetic properties can be used in MRI. Metal oxide NPs can be used for the production of positron-emitting tomography probes. Silica nanospheres (100 nm) along with agarose (1–2.5%) can be used in high resolution of ultrasound imaging. Nanoparticles can be used to amplify the contrast which can decline the exposure time of the body with harmful computed tomography contrast agents. Gold and bismuth can be preferred over iodine-based contrast agents as they can produce high contrast and decrease radiation exposure of patients.

Nanoparticles can be used for the treatment of a disease or drug delivery in cancer, inflammatory diseases, cardiovascular diseases, and wound healing. Chemotherapy and radiation therapy along with surgery are used for the treatment of cancer. Radiation therapy has the following limitations: (a) it can injure the surrounding cells, (b) sometimes tumor cells receive the lower intensity of radiation due to the distance of the site from the source of radiation, and (c) tumor cells can develop resistance for radiations. Radiation therapy can be improved by enhancement of radiosensitization of tumor tissues, radioresistance of healthy tissues, and reversion of radiation resistance in tumor tissue. Conjugation of nanoparticles with drugs can increase the toxicity of drugs for the treatment of leukemia. Attachment of AuNPs to drugs can enhance the efficiency of drug delivery and induce mitochondrial cell death.

Various NPs like gold half shell multifunctional NPs, PLGA NPs, and SLN have been utilized for the delivery of drug at the inflammation site in inflammatory diseases. Nanoparticles like AuNPs, PLGA, infliximab and MTX, actarit-loaded SLN, and gold half shell NPs have been used for the treatment of inflammatory diseases. Polylactic-co-glycolic acid-based NPs have been used for the delivery of drugs to the colon in inflammatory bowel diseases. Poloxamer encapsulated in PLGA can be

utilized to cure sodium arachidonate-induced inflammation in rabbits. Curcumin encapsulated in biodegradable microspheres, liposomes, hydrogels, biodegradable NPs, and cyclodextrin can be used for delivery of drugs at the inflammation site for the treatment of RA.

Gold nanoparticles can be utilized as antimicrobial, anti-inflammation, and antioxidants for skin burns and wounds. Synthesized AuNPs were used for the closure of wounds in rats. Methylcellulose gel with AuNPs was utilized in wound healing of surface burns. Wound healing process was determined by labeling of AuNPs with fluorescent agents. Epigallocatechin-3-gallate conjugation with NPs can be used as a therapeutic approach to cardiovascular diseases. Immune resonance scattering was used to determine the level of ApoAI and ApoB which are the risk indicators in cardiovascular diseases. Blood-brain barrier stops the delivery of drugs or unwanted compounds to the brain. Drugs conjugated with NPs have been used to deliver the drugs in the brain. The crossing of blood-brain barrier depends on the size, coating, and surface charge of NPs. Coatings of NPs to antimicrobial agents can enhance the properties of these agents, and these compounds can be used for broad spectrum for Gram-positive as well as Gram-negative microorganisms. Polycaprolactone scaffolds conjugated with AuNPs were used to repair myocardial infarction.

Nanomaterials like metals, zeolites, carbon nanotubes, fibers, metal oxides, and titanium oxides can be used to detoxify or transform the harmful compounds into nontoxic compounds. Nanotechnology is used to increase security in manufacturing, processing, and tracking of shipping products and preservation of food products for a long time. Silver is used as antimicrobial agents to decrease the bacterial growth for the preservation of food. Nanopackaging is used to increase the shelf life of enzymes, antioxidants, nutraceuticals, and antimicrobials in packaging. Extra 5 nm edible coatings were added on foods such as vegetables, candies, French fries, and chocolates for preservation. Biodegradable plastics such as polybutylene succinate (PBS), polylactic acid (PLA), and polyhydroxybutyrate (PHB) can easily be degraded to nontoxic compounds in the presence of microorganisms. Nano-TiO_2 with seeds can increase the absorption of inorganic nutrients and can quench the oxygen free radicals produced during photosynthesis which further can increase the germination and growth of spinach seeds. Sodium dodecyl sulfate (SDS)-modified photocatalytic TiO_2/Ag nanomaterial conjugated with dimethomorph (DMM) can be used as nano-pesticide to enhance the agricultural production. Nano-pesticide delivery techniques such as nanocages, nanoencapsulation, nanoemulsions, and nanocontainers are used for the protection of plants. Nanoparticles like parathion, methyl parathion, pirimicarb, fenitrothion, and paraoxon can be used for the detection of pesticide residues in plants or soil. Nanoparticles are also used as biomarkers for the detection of viral, bacterial, and fungal pathogens in agriculture.

In the last decade, scientific research has been focused on the usage of NPs for human welfare, i.e., biomedical applications, agricultural, nanoremediation, and food processing. The roles of several new NPs like AgNPs, AuNPs, and metal oxides in disease diagnosis, drug delivery, and food industry have been determined. There is a need for new approaches or techniques along with NPs which can diagnose the diseases efficiently. New biodegradable NPs are required for biomedical

applications and therapeutic approaches. Advanced technology has to be utilized for the encapsulation of drugs in NPs so that they can release the drugs at the site of infection. Further, new conjugates of NPs are needed for the preservation and security of foods. Nanoparticles have to be used as a broad spectrum in agriculture to increase the agricultural production and detection of pesticides or diseases in plants.

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Chapter 2
Nanotechnology: A Potential Tool in Exploring Herbal Benefits

Suddhasuchi Das and Amit Baran Sharangi

Abstract Nanotechnology is a novel approach that involves materials and equipment with manipulative abilities on the physical and chemical properties of a substance at molecular levels. The nanoparticles, so-called "mesoporous," introduce the gene and activate it in a precise and controlled manner without any toxic aftereffects. Polymeric nanoparticles, liposomes, proliposomes, solid lipid nanoparticles, and microemulsions are the precise nanoparticulate formulations which have a potential to deliver herbal medicines effectively. Herbal medicines have been widely used all over the world since prehistoric times and have been recognized by physicians for their better therapeutic value for having fewer adverse effects compared with modern medicines. The effectiveness of many species of medicinal plants depends on the supply of bioactive secondary metabolites. Most of these constituents of extracts, such as flavonoids, tannins, and terpenoids, are highly soluble in water but have low absorption, because they are unable to cross the lipid membranes of the cells, with bigger molecular size, resulting in loss of bioavailability and efficacy. To characterize the nanoparticles with a view to determine the physical and chemical toxicity profiles, several research studies are on their way to focus and explain the mechanism rationally aided with advanced technologies. Nanosized drug delivery systems of herbal drugs have a potential future for enhancing the activity and overcoming problems associated with plant medicines. The present chapter highlights several areas of this amazing world and focuses on the aspects of a promising fusion of nanotechnology and herbal wonders, converging into a futuristic wisdom.

Keywords Health · Herbal medicines · Medicinal plants · Nanoparticles · Nanotechnology

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_2

2.1 Introduction

The science of herbal medicines "Ayurveda" deals with herbal medicines/drugs and herbo-mineral preparations and is contributing since ages. The effectiveness of such treatments relies on standardized herbal products of consistent quality and well-defined constituents. Any herbal formulation to be pharmacologically functional is dependent on its phytochemical constituents present therein. It has been observed in few previous studies that the nanotechnology-based herbal drugs have improved solubility and enhanced bioavailability (Rasheed et al. 2012). The word "nano" means particles in the order of scale of 10^{-7} cm or 10^{-9} m. The technology essentially recognizes the particles less than the size of 100 nanometers (nm) and explores their new properties and behavior for potential utilization. In addition, "nanotechnology" is also a process which underpins the process of manipulation of individual atoms and molecules using set of precise tools to smaller molecules at a needed scale. The nanotechnology has become one of the most promising fields in recent years with capabilities of giving us many breakthroughs which might bring technological advances for a wide array of applications. The emersion of nanotechnology platforms would lead to development and commercialization of a wide range of bioactive macromolecules which will likely to have the great clinical implications in the near future (Farokhzad and Langer 2009).

This chapter focuses on the use of nanotechnology for "phytotherapy" or treatment of various diseases by herbal medicines/drugs, including herbal drug delivery using evolving nanotechnologies to enable entirely novel classes of therapeutics. The classical chemical methods for nano-herbal preparations have proven to be hazardous and not eco-friendly. However, the biological methods make use of microorganisms to produce nanoparticles as these products are nonhazardous in terms of human applications (Prasad et al. 2016). Because the synthesis of nanoparticles by using microorganisms is elaborative and time-consuming, producing nanoparticles by using herbal extracts is more preferred (Prasad 2014). Some researchers from Iowa State University used nanotechnology as a powerful implication for biotechnology to insert and activate the genes targeting into plant cell walls. The process of delivering genes into plant tissue occurs in two stages. The gene is first inserted into plant cell tissue followed by introduction of chemicals into plant to trigger the gene function.

Isolation and extraction of natural products (or herbal drugs) is full of technical hurdles. In spite of this, natural products isolated from plants have occupied a significant place in drug market. Visible positive effects with no side effects have made natural products a powerful therapeutic solution to the organisms, but poor solubility, poor permeability, low bioavailability, instability in biological milieu, and extensive first-pass metabolism create problems in the delivery of plant origin therapeutic molecules as drugs. Nano-encapsulation by attaching or encapsulating these therapeutics with suitable nanomaterials can be a better alternative to overcome these limitations. The pharmacokinetics and therapeutic index of plant origin drugs can significantly be enhanced by the nanomaterials with targeted delivery and combination therapy (Kumari et al. 2012).

2.2 Types of Nanoparticles

The various types of inorganic particles, namely, magnetic, metallic, ceramic, and nanoshells, their description, size, advantages, disadvantages, and applications are shown in Table 2.1. The organic nanoparticles include carbon nanotubes, quantum dots, dendrimers, liposomes, and polymers as described in Table 2.2.

2.3 Tools of Nanotechnology

Cantilever is a tiny bar which possesses the ability to bind to alter DNA protein associated with cancer (Garnett and Kallinteri 2006). Nanopores allow DNA to pass through one strand at a time; hence, they make DNA sequencing more efficient, and the shape or electrical property of each base on the strand can be monitored simultaneously. These properties are unique for each of the four nitrogenous bases which make up the genetic code. The nanopores can be used to decipher the encoded information including error in code associated with cancers. The molecule forming a hollow sphere, ellipsoid, or tube composed entirely of carbon is fullerene. There are spherical fullerenes which are called as buckyballs, while the other ones are cylindrical, known to be carbon nanotubes or buckytubes.

Nanotubes are smaller than nanopores. These are about half of the diameter of a molecule of DNA. These are first tagged with bulky molecule to exactly find out the point location of mutations associated with cancer. Dendrimers are macromolecules giving resemblance of a tree in a way that branching begins from the central core

Table 2.1 Inorganic nanoparticles

Inorganic compound	Description	Size range (nm)
Metallic	Gold and silver particles	<50
Magnetic	Super paramagnetic iron oxide particles	5–100
Nanoshells	Dielectric silica core in a thin gold metal shell	10–300
Ceramics	Inorganic porous biocompatible materials	<100

Table 2.2 Organic nanoparticles

Organic compound	Description	Size range (nm)
Carbon tubes	Cylindrical graphite sheets	1.5–5000
Quantum dots	Semiconductor crystals with a cadmium core and metal shell	<10
Dendrimers	Highly branched macromolecules	5–20
Liposomes	Phospholipids	5–100
Polymers	Colloidal particles	10–1000

and are constructed around a simple core unit. These are of high molecular uniformity with a narrow molecular weight distribution and specific in size and shape. They possess a central initiator core, and manufacturing process consists of a series of repetitive steps. The succeeding growth steps represent a new "generation" of polymer having larger molecular diameter with twice the number of reactive surface sites and approximately double in molecular weight than preceding generation (Garnett and Kallinteri 2006; Maureen and Val 2006).

2.4 Commercialization of Herbals

Commercialization efforts in herbal medicine by nanotechnology have now started around the world. Of the 300 companies identified being actively involved in nanoformulation worldwide, 259 are start-ups and small- and medium-sized enterprises (SME) that focus on the development of nanotechnology-enhanced phytopharmaceuticals and medical equipments and devices. Further, 41 other major pharmaceutical and medical device corporations have nanomedicine products on the market or run development projects in which nanotechnology plays a vital role (Paul et al. 2011; Kamboj 2000). Over the past decade, 38 nanotechnology-enhanced medical products were placed on the market with estimated total sales of EUR 5.4 billion in 2004 (Cott 1995; Sapna and Ravi 2007).

2.5 Herbal Medicines and Nanotechnology

Since ancient times herbal medicines are being widely used around the world. Herbal medicines that have been used in India are termed as "Ayurveda." This Indian medical science uses herbs or a mixture of herbo-mineral preparation. The seven therapeutic metals used in Ayurveda are gold, silver, copper, iron, lead, tin, and zinc. These metals are passed through various processes and are transformed into their therapeutic form.

Herbal drugs occupy a leading position in the pharmaceutical industry as their effects are known and no side effects are present. However, the delivery of these herbal therapeutic molecules as drugs is difficult due to its low solubility, poor permeability, instability in biological environment, and low bioavailability. These limitations can be overcome by encapsulating or attaching them with materials known as nanomaterials.

Nanotechnology is the advanced scientific technology of the twenty-first century. The term is derived from a Greek word "nanos" meaning dwarf. It is a new technology in drug delivery system. Nanotechnology makes use of nanoparticles that have a high surface area and can reach the targeted site because of its extremely small

size. Nanotechnology and herbal science is combined to overcome the limitations of using herbal drugs. The development of novel drug delivery system for herbal medicines includes nano-dose which helps in enhancing the biosolubility and bioavailability, protection from toxicity, and sustained delivery. Such novel drug delivery systems have site-specific action and predetermined rate.

Nanocarriers are made from safe materials, including biodegradable polymers, lipids, and polysaccharides. In novel drug technology, control of drug distribution is achieved by incorporating the drug into nanocarriers. Compared to micrometer-sized carriers, nanocarriers provide more surface area and increase solubility and bioavailability and enable exact drug targeting. As a result due to specificity, the amount of drug required to be incorporated to nanocarriers is much less than required when encapsulated. This is very useful when using expensive phytomolecules. Optimal use of phytomolecules improves cost-effectiveness of the product.

2.6 Need of Nanosized Delivery System for Herbal Drugs

Various herbal nanoparticle delivery systems have been summarized in Table 2.3. There are many setbacks of traditional herbal drug delivery systems which led to the emergence and application of newer nanosized herbal delivery system. Fewer reasons are mentioned below:

Table 2.3 Some herbal drug nanoparticles

Formulations	Active ingredients	Biological activity	Method of preparation
Curcuminoids solid lipid nanoparticles	Curcuminoids	Anticancer and antioxidant	Microemulsion technique
Glycyrrhizic acid-loaded nanoparticles	Glycyrrhizic acid	Anti-inflammatory antihypertensive	Rotary-evaporated film ultrasonication method
Nanoparticles of *Cuscuta chinensis*	Flavonoids	Hepatoprotective	Nanosuspension method
Pacli-taxe-loaded nanoparticles	Lignans, taxel	Antioxidant effects anticancer	Emulsion solvent evaporation method
Artemisinin nanocapsules	Artemisinin	Anticancer	Self-assembly procedure
CPT-encapsulated nanoparticles	Camptothecin	Anticancer	Dialysis method
Berberine-loaded nanoparticles	Berberine	Anticancer	Ionic gelation method

Ansari et al. 2012

- Nanoparticles enable targeted action of herbal medicines toward a specific organ which thereby improves the effectiveness and safety and reduces the dosage.
- Improved herbal drug solubility and localized action of drug on a specific site, thus resulting in better efficacy.
- Their unique size and high loading capacities help them able to deliver high concentrations of drugs to disease sites.
- Small particle size of drugs enhances the entire surface area of the drugs, therefore increasing the delivery and allocating quicker dissolution in the blood.
- Shows enhanced permeation effect due to the small size and retention due to poor lymphatic drainage such as in tumor.
- Reduced incidence of side effects.

2.7 Sources of Nanoparticles from Herbal Plants

Nanoparticles of various sizes and shapes can been synthesized from different parts of plant like the stem, root, leaves, fruit, and seed.

2.8 Process of Synthesis of Nanoparticles

2.8.1 The Classical Method (Bottom-Up Method)

The classical method of synthesis of inorganic nanoparticles includes wet-chemical methods. In this method, a chemical reaction is carried out by mixing suitable reagents which lead to a solid phase. Solid particles of 1–2 or less nm are formed. This is known as nucleation. By thermodynamically favored reaction, these small crystals are grown into larger crystals. The colloidal stability can be due to electrostatic repulsion or steric stabilization, or both.

2.8.2 The Advanced Method (Top-Down Method)

The latest research in nanotechnology is focused on nanosizing herbal extracts and takes diversified advantages. Different methods are used to make nanoparticles by using herbal extracts. These include the following:

- High-pressure homogenization method.
- Complex coacervation method.
- Coprecipitation method.

- Salting-out method.
- Solvent displacement method.
- Super critical fluid method.

Much safer methods are also used to synthesis of metallic nanoparticles from herbal extracts, and these processes are known as green synthesis. These are as follows:

- Polysaccharide method.
- Irradiation method.
- Biological method.
- Polyoxometalate method.
- Tollens method.

2.9 Disadvantages of Biosynthesis of Nanoparticles from Herbal Extracts

The change in the physicochemical and structural properties of nanosized materials due to size decrease could result in a number of material interactions that may lead to toxicological effects.

2.10 Standardization of Nano-Herbal Medicines

Herbal product needs proper scientific validation, authentication, and characterization to ensure reproducibility in its manufacturing process (Vaidya and Devasagayam 2007). Hence, standardization is necessary for herbal medicines. Standardization ensures a predefined amount of quantity, quality, and therapeutic effect of ingredients in each dose (Zafar et al. 2005). The therapeutic activity of any herbal formulation depends on its phytochemical constituents. To ensure reproducibility as a unique quality of herbal medicine, the authentication of herbal drugs and identification of adulterants from genuine source are essential for both pharmaceutical companies and public health (Straus 2002).

Incorporation of nanotechnology in herbal medicines increases solubility, stability, bioavailability, and pharmacological activity of many popular herbal extracts including *Ginkgo biloba*, grape seed, green tea, and ginseng. This can be achieved using nano-dosage forms previously reported such as polymeric nanoparticles, nanospheres, nanocapsules, liposomes, proliposomes, solid lipid nanoparticles, and nanoemulsions (Huang and Chang 2009). On the other hand, herbal nanomedicine includes protection from toxicity, improving tissue macrophages distribution, sustained delivery, protection from physical and chemical agents, etc. Furthermore, to standardize herbal drugs, various advanced methods

such as chromatography, spectrophotometry, chromatography-spectrophotometry, electrophoresis, polarography, and molecular biomarkers are currently used (Seitz et al. 1991; Svicekova et al. 1993; O'Shea 1995; Mosihuzzaman and Choudhary 2008).

2.11 Advanced Techniques for Identification and Characterization of Nano-Herbal Medicine

2.11.1 HPLC

Preparative and analytical HPLC are being widely used in pharmaceutical industry for isolation and purification of herbal compounds (Chimezie et al. 2008; Saravanan et al. 2010). Vasicine, the major bioactive alkaloid in *Adhatoda vasica*, is found to be rich in two polyherbal drug formulations—Shereeshadi Kashaya (18.1 mg/100 g) and Yastyadivati (0.7 mg/100 g) (Anupam et al. 1992). Standardization of the Triphala (an antioxidant-rich herbal formulation) mixture of *Emblica officinalis*, *Terminalia chebula*, *and Terminalia belerica* in equal proportions has been reported by HPLC method by using the RP18 column with an acidic mobile phase (Singh et al. 2007). At present, the most powerful technique for the quality control of Chinese herbal medicine Gan-Cao (licorice) is the combination of HPLC and LC/MS (Zhang and Ye 2009).

2.11.2 HPTLC

HPTLC technique is being widely used in pharmaceutical industry for process development of nano-herbal drug formulations and identification and detection of adulterants/pesticides in herbal products, and it also helps in identification of mycotoxins and thus authenticates the quality control of herbs and health foods. HPTLC technique was reported for simultaneous determination of withaferin A and β-sitosterol d-glucoside in four ashwagandha formulations (Soni and Naved 2010). *Syzygium jambolanum* was quantitatively evaluated in terms of stability, repeatability, accuracy, and phytoconstituents such as glycoside (jamboline), tannin, ellagic acid, and gallic acid by HPTLC (Shanbhag and Khandagale 2011) (Table 2.4).

2.11.3 UPLC

Ultra-performance liquid chromatography (UPLC) was used to evaluate decocting-induced chemical transformations and chemical consistency between traditional and dispensing granule decoctions (Mike and Edward 1999; Li et al. 2010a, b).

Table 2.4 List of phytoconstituents modified by nanotechnology

Approach	Experimental work done	Significance
Curcuminoids	Enhance curcuminoid bioavailability and reduce perceived toxicity	Improved cellular uptake, enhanced dissolution rates, excellent blood stability, controlled release functions, multifunctional design, enhancement in its pharmacological activities (e.g., antioxidant and antihepatoma activities)
Berberine hydrochloride	Anticancer activity and novel mechanisms have been explored, the chance of regulating glucose and lipid metabolism in cancer cells showing more potential than ever	In recent years, the pharmaceutical preparation of berberine hydrochloride has improved to achieve good prospects for clinical application, especially for novel nanoparticulate delivery systems
Colchicine	The effect of eugenol on intestinal absorption of colchicine in an oral administrative nanoemulsion formulation	Relative bioavailability of nanoemulsion and eugenol nanoemulsion were enhanced by about 1.6- and 2.1-fold
Genistein	Newer formulations of genistein such as diindolylmethane (BDIM) from BioResponse Inc. has shown some enhanced bioavailability	Genistein has been shown to possess anticancer activities
Resveratrol	Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a phytoalexin produced naturally by several plants when under attack by pathogens such as bacteria or fungi	The earliest reported nanoformulation of resveratrol comes from a study by Yao et al., where they prepared resveratrol chitosan nanoparticles with free amine groups on the surface so as to conjugate ligands, which will actively target to special tissues or organs

2.11.4 LC-MS

Previous researches showed that LC-MS analyses of aminoglycoside drugs are highly soluble in water, exhibited low plasma protein binding, and were more than 90% excreted through the kidney. Further this technique helped in analysis of amino glycosides in plasma samples with ion pairing chromatography (Shen et al. 2010).

2.11.5 GC-MS

It is the system used for identification of large number of components present in natural and biological systems (Binit et al. 2010). It has been used by several researchers for the identification and quantification of chemical constituents present. Likewise, Kasthuri et al. (2010) isolated *Myristica fragrans*, *Eucalyptus globulus*, *Gaultheria procumbens*, and *Mentha piperita* from polyherbal oil formulation

(Megni) by GC-MS method. Thirty-five volatile compounds were separated and identified (Shaa et al. 2004).

2.11.6 Capillary Electrophoresis

The methodology of CE was established to evaluate herbal drugs in terms of specificity, sensitivity, and precision (Ganzera 2008). Several studies based on electrophoresis analysis of herbal medicines have been reported where alkaloids (Wen et al. 2005) and flavonoids have been studied extensively (Pietta et al. 1991). Additionally, the analysis time of the CE method was reported to be shorter than HPLC and with 100-fold less solvent consumption (Sombra et al. 2005).

2.11.7 Atomic Force Microscopy (AFM)

The AFM probe interacts with the substrate through a raster scanning motion with a very high resolution type of scanning probe microscopy. The resolution is on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit (Lang et al. 2004). AFM provides a three-dimensional surface profile (Geisse 2009). AFM images revealed uniform nanofibers present in relatively high abundance in a solution of the medicine, Yunnan Baiyao, a traditional Chinese herbal medicine that is being used to treat wounds for over 100 years.

2.12 Nanotized Herbal Medicines in Some Conditions/ Diseases

The application of nanotechnology in medicinal area or more specifically drug delivery is being implemented rapidly. The positive aspects of nanosized drugs are captivating pharmaceutical sciences to use nanoparticles which additionally possess the potential to cross the blood-brain barrier and open ways for drug delivery into the brain. Multiple substances are currently being investigated for the preparation of such nanoparticles. These include protein and lipid based, i.e., albumin, gelatin, and phospholipids for liposomes, and few chemical based like various polymers and solid metal-containing nanoparticles (Jong and Borm 2008).

The research on herbal remedies is being carried out all over the world in different areas, e.g., pharmacy, pharmacology, and medicine. Unlike allopathic system, the herbal drugs are also functional due to mechanism of action of hundreds and thousands of constituents all together against diseases. The conventional pharmaceuticals available in market are derived from natural products which are playing a vital role. These natural products are produced by the organisms like fungi, bacteria,

animals, and plants. In early years, drugs such as aspirin, salbutamol, digoxin, quinine, morphine, atropine, colchicine, and bromelain were made from natural plant extracts and are now known as active components for treating various diseases. Morphine, isolated as a first drug by Serturner from opium poppy (*Papaver somniferum*), acts as a pain killer. Cinchona tree extract, quinine, is used to treat malaria, and aspirin isolated from willow bark is used for treatment of fever. Most of the plants and formulations (e.g., curcumin, Triphala, pomegranate, Kalonji, Sariva) have been explored for their potential to cure cancer and inflammation (Yadav et al. 2011).

Incorporation of these herbal extracts into novel formulation systems is yet to be done in order to overcome bulk dosing and less absorption which are the major problems being faced. Major natural products and their derived active components present in the market are paclitaxol and doxorubicin (both anticancer drugs); lovastatin (anticholesterolemic); erythromycin, streptomycin, and rifamycin (all antibiotics); cyclosporine-A and tacrolimus (both immunosuppressive); and amphotericin-B (fungicidal).

Few researchers developed a new drug from anticancerous potential Chinese herbs like milk vetch root, saltwort, cassia twigs, and liquorice root. The herbal drugs were able to target cancer cells without damaging the healthy human cells. The drugs passed first phase of animal testing and possess patent rights of 42 countries. It is advantageous in terms of killing only cancerous cells, rapid medicinal effects, and it causes no harm to other organs. Together with Western medicines, it lowers the dosage and reduces resistance to cancer drugs, thereby enhancing overall efficiency. It was also found to be effective in alleviating symptoms of the cancers of the lung, breast, bone, liver, tongue, cervix, ovary, brain, and skin and primary or metastatic lymphoma (Garg 2010).

Enhanced solubility and permeability effects could be achieved through highly stable micelles of size 10–40 nm prepared from poly(ethylene glycol)/phosphatidylethanolamine (PEG-PE) conjugates. Due to high retention efficiency of significant quantities of poorly soluble anticancer drugs (m-porphyrin, tamoxifen, Taxol), they represent a convenient drug delivery system into tumors. Development of nanosized dosage forms of camptothecin-derived drugs improved this drug efficiency in tumor. Plant viruses are also being drafted for drug delivery in tumors using nanotechnology. These viruses have been successfully modified by researchers to deliver drugs only to specific cells inside the human body, without affecting surrounding tissues. These are tiny "smart bombs"—a thousand times smaller than the human hair—and have the ability to survive outside plant host and its built-in "cargo space" of 17 nm, which can be used to carry chemotherapy drugs directly to tumor cells. Among different nanoparticles to be used as cell-targeting vectors, the plant virus is superior in terms of various aspects including stability, targeting cells, and carrying therapeutic cargo. Calcium keeps the virus' cargo enclosed so that when the virus is in the bloodstream, the lower calcium levels inside the cells would allow the virus to open, thus delivering the drugs only to the targeted cells. The toughness of shell makes the virus unique, and after it opens, it gets time to enter the cell nucleus which increases the drug's efficacy. Nanoparticles can be

attached to a small RNA molecule to initiate virion assembly forming virus-like particles smaller than native virus. Several metals are used in Ayurveda for therapeutic reasons, majorly, seven metals, viz., gold, silver, copper, iron, lead, tin, and zinc. These metals are processed to yield "bhasma," a metal-based medicine. The bhasma may also be called as metalomedicine containing nano- to submicron-sized particles, e.g., particle size of "Swarna bhasma" (gold ash) is about 56 nm. By repeated incineration and grinding with some herbal juices and other specified matters, raw metal is converted into therapeutic form through the classical process. The conversion of raw metal is carried out using mechanical processes not by chemical ones (Garg 2010).

Phytotherapy is termed by chemists for the use of nanotechnology for plant research. Numerous nanoparticles occur naturally in the environment; however, "mesoporous" nanoparticles introduce and activate the gene at the same time, in a precise and controlled manner and without toxic after effects. The nanoparticles are chemically coated with a container-like action for the genes to be delivered to the plants. The coating induces the plants to swallow the particles, effectively ingesting them inside the plant cell walls, where the genes could be inserted. The successful attempts of these techniques are tobacco and corn plants. The alterations in plant cell wall with the aid of nanotechnology enable researchers to instantly impart any substances to the plants and release in a time-controlled manner. This would enable biologists to view the complex world of plants in their intricate details. Several research initiatives extend scope of application for a range of plants with possible early detection of plant diseases as well (Garg 2010).

By using the aqueous extracts of *Aloe vera*, ginger (*Zingiber officinale*), garlic (*Allium sativum*), tulsi (*Ocimum tenuiflorum*), oils (e.g., coconut oil), antifungal drugs (e.g., fluconazole and itraconazole), and antibacterial antibiotics (e.g., tetracycline and chloramphenicol), silver nanoparticles have been synthesized from *Lactobacillus acidophilus* 01 strain. The synthesized silver nanoparticles, together with plant products, showed inhibitory effect against clinical isolates. This implicates that the biologically synthesized nanoparticles in combination with certain plant products give better chemotherapeutic effects against microbial diseases (Raja et al. 2011).

In Africa, *Artemisia annua* plants are used as "phytotherapy" to treat malaria. The commercialization is limited due to lower yield (0.01–0.8%) of artemisinin from wild *A. annua* plant. However, it could be semisynthetically produced from precursor artemisinic acid which is present in excess amounts (tenfold) in the plants. Chemical synthesis of artemisinin-like endoperoxides, e.g., arteflene, preserves the natural resources of *A. annua* plants. Phytotherapeutical, agricultural, and biotechnological approaches could be better ways to meet the high demand for artemisinin. These approaches allow the cultivation of wild plants in fields and greenhouses. The breeding of high-yield cultivars and the cultivation of transgenic plants are done. Genetically modified organisms, i.e., *E. coli* and *Aspergillus flavipes* or *Saccharomyces cerevisiae*, have been reported to produce 25-kg artemisinin in a biosynthetic pathway. Another approach is in vitro culture of *A. annua* to produce

hairy roots through infecting roots with *Agrobacterium rhizogenes*. These hairy roots produce significant amounts of secondary metabolites including artemisinin. Application of sophisticated biotechniques to the production can meet the high demand for artemisinin for malaria as well as cancer chemotherapy.

2.13 Nanophytomedicine: Nanotechnology in Herbal Drugs

The use of nanophytomedicine shows improved efficacy and bioavailability of administered drugs and also decreases the side effects and toxicity of administered drugs.

2.13.1 Anticancer Therapy

Nanoparticle surface modified with cationic chitosan is efficient for drug delivery both in vitro and in vivo. Breast cancer is already being treated by approved drug Abraxane which is a nanoformulation (paclitaxel) adhered to nanobead protein structure. The nanobead protein-conjugated formulation increases water solubility, allowing for elimination of the toxicity associated with the solvent vehicle (cremophor) and improved therapeutic index. Veteh root-, seawort-, cassia twig-, and liquorice root-extracted nanotized herbal drugs are found to be effective in pulmonary, liver, bone, brain, and skin cancer. This nanoformulation enters the cancerous cell without damaging the healthy cell (Maeda and Matsumura 1989; Stolniket et al. 1995; Schnitzer 2001; Gabizon et al. 2003).

2.13.2 Nanocurcumin

Curcumin is a fat soluble molecule which has poor water solubility, greatly limiting its bioavailability. Curcumin also metabolizes quickly, further reducing its bioavailability. These problems have led to search for a more usable, beneficial, and bioavailable super-curcumin which is prepared by tiny particle of curcumin encapsulated in oil cavity surrounded by membrane. Encapsulation in these tiny particles allows lipid soluble curcumin to be better absorber and also slows the release into bloodstream, enhancing and improving bioavailability. In vitro and animal studies have elucidated that curcumin may have antitumor, antioxidant, antiarthritic, and anti-inflammatory activities. The bioavailability of curcumin increases ninefold when administered as entrapped nanoparticles as revealed by in vivo pharmacokinetic studies (Anand et al. 2008).

2.13.3 Green Nanotechnology (Cumin-Mediated Gold Nanoparticle)

To stop nanoparticles from agglomeration, several toxic chemicals in the form of either reducing agent to reduce various metal salts to their corresponding nanoparticle or stabilizing agents are in use. Hydrazine and sodium borohydride, two highly toxicones, are being used to produce gold nanoparticles and various reduced metals for nanophytomedicine, providing inherently green approach to nanotechnology referred as green nanotechnology. The antioxidant characteristics cumin resides in few functional groups such as carboxyl, amino, thiol, and hydroxyl including cuminaldehyde, alpha and beta pinene, cuminin alcohol, p-cymine, and beta terpene which also possess reduction power for producing gold nanophytomedicine. Experimental studies have shown that combination of the chemicals along with gum arabic was responsible for synthesis of gold nanophytomedicine in aqueous medium (Kavita 2009).

2.13.4 Ayurvedic Bhasma: A Nano-Preparation

Bhasma is an ancient but ultra-modern nanomedicine prepared from metal raw material through a series of scientific process (viz., repeated incineration and grinding with some herbal juice and other specified drugs) into the therapeutically active form. Due to its small size, the basic character gets changed due to the corresponding changes in electrical, thermal, inorganic, optical, chemical, and biological behavior. Swarna bhasma is a therapeutic form of gold metal of nanosized particle. When evaluated by various tools and techniques like AFM (atomic force microscope) and SEM (scanning electron microscope), it was found that the size of particle was 56 nm. Analysis by FT-IR and XRD shows that pure gold is in zero valence state (Sarkar and Chaudhary 2010).

2.13.5 Nanotechnology in Cosmetics

Nanoparticles in personal care product sit on top of the skin care formulation. Nanoparticle in sunscreen has been used safely and effectively by consumers for decades to protect from harmful UV rays and skin cancer. ZnO and TiO_2 are also important ingredients of nanophytomedicine which can provide great protection from the sun.

2.13.6 Aloe vera *Extract in Nanoparticle*

Aloe vera extract is widely used for skin care mostly in dermatitis, psoriasis, dryness, scaling, flaking, eczema, sunscreen lotion, and antiaging preparations. It has been reported by Japanese scientists in a recent study that *Aloe vera* cannot penetrate stratum corneum of the skin. They have investigated that liposome-containing *Aloe vera* from soybean lecithin could enhance penetration. *Aloe vera* that contained liposome within having a diameter less than 200 nm were found to penetrate better during in vitro test using human skin fibroblast and epidermal keratinocytes. According to study cell proliferation rate was significantly higher with the liposomal *Aloe vera* than nonencapsulated. In addition collagenase synthesis has increased by 23% with liposomal extract compared with 4% nonencapsulated extract (Makoto et al. 2009).

2.14 Recent Developments

The nanoparticles now have become as the capable approach in the drug delivery systems for the well-organized delivery of herbal drugs utilized in the treatment of many critical diseases such as cancer by crossing the reticuloendothelial system, increased permeability and retention effect, and tumor-specific targeting (Bombardelli et al. 1994). Recently, pharmaceutical researchers have shifted their focus to designing a drug delivery system for herbal medicines using a modern approach. *Cuscuta chinensis*, a traditional Chinese medicine, is used to nourish the liver and kidney, but the oral absorption of drug is limited by its chemical constituents such as flavonoids and lignin (Bombardelli 1991; Kakkar et al. 2016). Hence, the nanoparticles for the same were developed (Bombardelli 1991; Kidd and Head 2005), and work has also been carried out in the development and characterization of SLNs for the traditional Chinese medicine for their targeted delivery and increased bioavailability and efficacy (Muller et al. 2000; Sahni et al. 2011). A recent experimental study of polylactic acid nanoparticles of lipophilic anticancer herb drug (cucurbitacins and curcuminoids) using a precipitation method has been developed (Fessi et al. 1989; Maiti et al. 2005). In the recent years, nanostructured carrier systems such as microemulsion, polymeric nanoparticles, liposomes, silver nanomaterials, SLNs, polymeric micelles, nanoemulsions, and microsphere have been discovered for their potential to deliver anticancer drugs by oral route (Barzaghi et al. 1990; Moscarella et al. 1993). Moreover, the oral route offers a great potential for delivery of cytotoxic agents, and therefore, the attention has focused on the development of oral dosage forms for chemotherapy in oncology (Min et al. 2008; Chen et al. 2009; Li et al. 2009).

2.15 Future Perspectives

Research on herbal remedies and natural products is an emerging area of interest globally. The basic need is to develop the better systems for the proper delivery of such drugs at the sites and in the whole body in the doses which will not compromise with the existing treatment. Herbs as nanocarriers will not only increase the potential for the treatment of various chronic diseases and health benefits but also drastically reduce the side effects. The collaborative research among the traditional "herbal remedies" and newer approaches of modern drug delivery system, i.e., "nanotechnology," has established the attractive therapies to the pharmaceutical in the near future that will enhance the health of people (Sethiya et al. 2010).

Nanotechnology offers various modern applications in novel drug delivery systems that potentially improve the diagnosis and treatment and help to monitor post-administration transformation of drug composition within the body systems. Computer-aided drug designalso offers a lot of scope for the development of this kind of novel and advanced systems and helps in designing and developing the drugs and delivery systems, consuming lesser time and resources with more accuracy and quality compared to traditional methods (Giacomazza and Carlo 2012; Karaman 2012; Pawar and Bhangale 2015).

Research on herbal remedies and nutraceutical products is more familiar worldwide. The development of phytomedicine in the drug delivery system in a number of organizations is being carried out at basic and clinical trial levels (Lin et al. 2007; Sethiya et al. 2010). To improve the proper delivery systems at the sites or locations in the whole body in a particular dose will not compromise with the existing treatment (Shakeri and Sahebkar 2016). This not only would give relief from unwanted effects such as toxicity and hypersensitive reactions but also will increase the patient's strength internally which is very much confidence boosting and desirable. In the future, the concept of herbal nanoparticles for the treatment of critical diseases such as cancer, diabetes mellitus, and anemia with drug delivery system may also fascinate some potential research groups and potentially create attention-grabbing results (Yen et al. 2010; Bonifacio et al. 2014).

2.16 Conclusion

Nanotechnology perfectly tuned with traditional herbal medicine may provide a very useful tool in designing future herbal medicine with improved bioavailability profile and less toxicity. The well-connected plant sciences and nanotechnology have the potential to develop an attractive symbiosis between green revolution and nanotechnology with realistic prospects for minimizing the application and toxic chemical generation, causing destruction to living organisms and our environment. Specific priority must be given to determine how to best manage nanotechnology safely and ethically.

Hence, using "herbal therapy" in the form of nanocarriers will definitely increase its potential for the treatment of many chronic diseases and health benefits. Natural remedies are also prosperous resources of advantageous compounds holding antioxidants and constituents that can be made useful in purposeful foods. This type of research among the traditional "herbal remedies" and newer approaches of modern drug delivery system, i.e., "nanotechnology," has the lucrative or attractive therapies to the pharmaceutical in the near future that will enhance the health of people. It is anticipated that the effective and valuable relevance of the natural products and herbal remedies being applied with the nanocarrier will enhance the significance of existing drug delivery systems.

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Chapter 3
Nanotechnology: An Effective Approach for Enhancing Therapeutics and Bioavailability of Phytomedicines

Zarith Asyikin Abdul Aziz and Siti Hamidah Mohd Setapar

Abstract Phytomedicines have been widely implemented throughout the world for centuries and recognized by scientific committees and patients for their better therapeutic value, with fewer adverse effects offered by herbal medicines compared with modern drugs. The enhancement of phytotherapeutics demands a scientific approach in delivering the biological constituent efficiently, in a sustained manner to achieve patient satisfaction and avoid repeated administration. Incorporation of phytomedicines into nanotechnology is an innovative approach to magnifying the solubility, absorption rate, and permeation membrane of phytomedicine, which possess the high bioavailability and therapeutic potential of these medicinal plants. Therefore, this chapter reviews numerous investigations regarding the pharmacological activity of phytomedicine, the main factors that limit the therapeutic potential of phytomedicine, and the incorporation of phytomedicine into several nanocarriers. Hence, the overall discussion has revealed the potential of novel drug delivery systems in intensifying the bioavailability and efficacy of the pharmacological activity of phytomedicine. This finding depicts the several in vivo assays of phytomedicine-loaded nanocarriers that have been discussed throughout this chapter.

Keywords Bioavailability · Nanocarriers · Nanotechnology · Phytomedicine · Therapeutic potential

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_3

3.1 Introduction

3.1.1 Phytomedicines

Knowledge and implementation of plants as herbal medicines, typically known as phytomedicines, have been introduced into various populations throughout human evolution, where people have started to prefer selecting plants for ailments, disease treatments, and foods (Aziz et al. 2016). Mainly in developing countries, almost 75–80% of the world population are maintaining their lifestyle by consuming herbal medicines for primary healthcare (Kamboj 2000). They believe that fewer adverse effects of drug consumption can be achieved by the usage of phytomedicines, as well as the cheaper and local availability of these drugs compared with conventional or modern drugs (Gupta and Raina 1998). According to the World Health Organization (WHO), the worldwide application of phytomedicines exceeds that of modern medicines multiple times. Besides, the ailment treatments for healing purposes using herbal plants predate human history and form the origin of many modern medicines (Pal and Shukla 2003).

Numerous reasons have been reported for patients' desire to use herbal therapies, where the mental comfort of taking action and a sense of control are often cited. With regard to this matter, people are taking herbs as an alternative to treating some ailments such as rheumatism, cancer, diabetes, cardiovascular disease, or AIDS (Pal and Shukla 2003). Frequently, herbs are being used as phytomedicines in home remedies for self-timing conditions such as sore throats, colds or bee stings, because professional care is not immediately available, is time-consuming, and is costly (Winslow and Kroll 1998).

In rural areas, there is an environmental culture, the so-called "man–earth relationship," an additional cultural factor that encourages the use of herbal plants where this culture makes people believe that when the area has an increasing amount of diseases, the plants can be applied to cure them (Winslow and Kroll 1998). There are many primary health centers, which are planned to serve rural areas with diagnostic facilities and adequate supplies of conventional drugs; however, residents of rural areas are still heavily dependent on traditional plant-based treatments (Mudur 1995).

A century ago, numerous conventional drugs were developed using plant sources, and a few effective drugs originate from a plant base. Examples of plant medicines used are willow bark, cinchona bark, foxglove, and opium poppy to produce aspirin, quinine, digoxin, and morphine respectively (Vickers and Zollman 1999). However, in proportion with the industrial revolution, the rise of allopathic medicines was revealed and the usage of herbal medicines was viewed less enthusiastically (Pal and Shukla 2003). Thus, in the mid twentieth century, most herbal products were discarded owing to political, economically profitable, and social changes (Aziz et al. 2016).

In the 1960s, the interest in "natural health" and the implementation of herbal products increased with regard to individual concerns over the adverse effects of allopathic medicines and a craving for more self-reliance. In 1992, the National

Institutes of Health, USA, established the Department of Alternative Medicine owing to the recognition of the rising use of phytomedicines and other non-traditional medicines. In addition, the WHO revitalized the residents among developing countries worldwide to use traditional plant medicines and herbal plants received a boost in demand (Winslow and Kroll 1998).

In 2003, the WHO claimed that traditional medicines, including phytomedicines, as constituting a therapeutic approach that is still in use today, even though they have been in existence for hundreds of years before the emergence of modern medicines. The earliest implementation of phytomedicine was recorded in Indian, Egyptian, Chinese, Roman, Greek, and Syrian texts dating back about 5000 years (Pal and Shukla 2003). From a scientific perspective, the enormous physicochemical, biological, and pharmacological activities of medicinal plants impress the scientific committees. Chemical constituents of phytomedicines and their popular implementation have become the research focus. Numerous researchers have portrayed and reported the pharmacological activities of phytomedicines from all over the world, describing the biological potential of medicinal plants that could benefit the pharmaceutical sectors.

Maregesi et al. (2008) revealed the antibacterial potential of medicinal herbs from Tanzania in inhibiting 12 microorganisms, including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (bacteria), *Candida albicans* and *Aspergillus niger* (fungi), herpes simplex, vesicular stomatitis, Coxsackie B2, and *Semliki Forest* A7 (viruses).

In addition, the anticancer pharmacological activity of phytomedicines has been demonstrated by Shoemaker et al. (2005), Faried et al. (2007), and Ghasemzadeh and Jaafar (2013). These investigations reported the in vitro cytotoxicity of *Pandanus amaryllifolius* Roxb., *Phaleria macrocarpa* (Scheff.) Boerl., and 12 Chinese medicinal herbs against several human cancer cell lines.

Another evaluation of the biological properties of phytomedicines, such as antioxidant, anti-inflammatory, analgesic, and antiviral, have been reported by researchers (Shin et al. 2006; Mohamed et al. 2010; Krishnaiah et al. 2011). With regard to these matters, interest in scientific investigation into phytomedicine is rising among scientists and there are the possibilities of the further implementation of phytomedicine in the development of pharmaceutical products.

3.1.2 Pharmacological Activities of Phytomedicines

There is a vast body of evidence for the pharmacological or biological properties of phytomedicines that are represented by the chemical constituents they contain. Most of the investigations showed the significant potential of herbal medicine to be further introduced and implemented in pharmaceutical formulations. The next subsections discuss the pre-clinical and clinical evaluations of the pharmacological activities of phytomedicines.

3.1.3 Antibacterial Activities

Phytomedicines tend to be known to be effective in treating some infectious diseases throughout the history of mankind. Since the discovery of antibacterial drugs, bacterial infections have been controlled remarkably effectively. However, some of the bacteria are reported to be resistant to the first effective antibacterial drugs discovered. In 2002, the WHO stated that there is a search for new antibacterial agents, in particular medicinal plants with regard to the development of drug resistance as well as the appearance of undesirable adverse effects (Pal and Shukla 2003).

Zaidan et al. (2005) investigated the antibacterial potential of commonly used phytomedicines in Malaysia, *Vitex negundo*, *Andrographis paniculata*, *Piper sarmentosum*, *Morinda citrifolia*, and *Centella asiatica*, against five species of bacteria: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli.* This in vitro evaluation was accessed using the disk diffusion method. The antibacterial activities were evaluated by measuring the minimum inhibition concentration (MIC) values of each medicinal plant. In addition, all the herbal plants were extracted by using a solvent extraction method using different solvents. Ethanol was used to extract the dried ground leaves of *Vitex negundo*, methanol for *Centella asiatica*, *Piper sarmentosum*, *Morinda citrifolia*, and dried leaves of *Andrographis paniculata* were macerated using a water solvent.

Depicting the MIC result, it was shown that only *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and MRSA were the most susceptible to the plant extract. *Andrographis paniculata* water extract had the most successful antibacterial potential against *P. aeruginosa* at an MIC value of 2 μg/disc. In addition, *Vitex negundo* showed no antibacterial activity against all bacteria strains and it was reported that this was due to the ethanol solvent used to secrete this medicinal plant, whereas numerous ethanol extracts of medicinal plants were revealed to be poor antibacterial agents (Zaidan et al. 2005).

Piper sarmentosum and *Andrographis paniculata* demonstrated the potential to inhibit *Staphylococcus aureus* and MRSA bacteria. *Staphylococcus aureus* is a common bacterial species that causes infections including food poisoning, localized abscesses, and superficial skin lesions, whereas MRSA infections mostly develop among patients admitted to hospital. As both the phytomedicines were reported to have potential against these dangerous bacteria, further evaluations of these plants are needed.

In addition, the other strains that were not susceptible to the plants extract are *Klebsiella pneumoniae* and *Escherichia coli.* This was elaborated owing to the common properties of Gram-negative bacteria, which the double membrane presence surrounding the bacterial cell, commonly known as the unique outer membrane. This outer membrane creates resistance to exclude certain drugs from penetrating the cell, including antibiotics (Zaidan et al. 2005). The Minimum Inhibitory Concentration (MIC) result is shown in Table 3.1.

In another study, the different segments (root, flower, leaf, and stem) of the methanol extract of a medicinal plant commonly planted in tropical or temperate Asian countries and Africa, known as *Leucas aspera*, was evaluated for its antimicrobial potential against Gram-negative and -positive bacteria: *Salmonella typhimurium*, *Escherichia coli*, *Salmonella aureus*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Salmonella enterica.* The antimicrobial activity of the plant extract was conducted by using the disk diffusion technique and the inhibition zone resulting from the extracted segments of each medicinal plant was recorded. The result was compared with that of commercialized antibiotic drugs, chloramphenicol (CP; positive control) and methanol (negative control) (Chew et al. 2012).

According to the findings, CP demonstrated larger bacteria inhibition zone ranges of between 21.0 and 25.0 mm, compared with the ethanol extract of all plant segments with the ranges of 7.0–11.0 mm. Among the plant segment extracts, the root extract was reported to be the highest zone of bacteria inhibition against all the examined microorganisms, followed by flower extract, which is highly sensitive to *Salmonella* species compared with the other species of bacteria. Leaf extract was potent in inhibiting four microorganisms. On the other hand, the negative control, methanol, did not inhibit any of the bacterial strains examined (Chew et al. 2012).

The successful antibacterial potential resulting from the commercial antibiotic drug, CP was may be due to the fact that the pharmacological constituents contained inside the plant extracts were formed in smaller concentrations (Zuraini et al. 2007). In addition, this study reported the effectiveness of plant extracts in inhibiting both negative and positive bacterial strains, which may be attributable to the presence of a broad spectrum of antibiotic constituents or simply general metabolic toxins (Srinivasan et al. 2001).

Furthermore, Sen and Batra (2012) demonstrated the antimicrobial activity of *Melia azedarach* L., a phytomedicine extracted by different solvents such as ethanol, methanol, water, and petroleum ether. These extracts were examined for their antibacterial potential against numerous human pathogens such as the bacteria *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *E. coli*, and the fungi *Rhizopus stolonifera*, *Fusarium oxysporum*, *Aspergillus niger*, and *Aspergillus flavus* by using an agar well diffusion method (Table 3.2).

The antimicrobial efficacy of plant extracts was compared with that of controls, ampicillin and fluconazole at 1.0 mg/disk for both. The results reported that all the medicinal plant solvent extracts are efficient at inhibiting all the microorganisms systems. Among the solvents implemented, ethanol and methanol were revealed to be the most potent antimicrobially and antifungally, followed by petroleum ether and water. The maximum inhibition zone was obtained by the medicinal plant extracted using ethanol, with a diameter 22.3 ± 0.42 mm and 19.5 ± 0.52 mm, against *P. aeruginosa* and *Staphylococcus aureus* respectively. Methanol plant extracts demonstrated a slightly smaller inhibition zone inhibiting *E. coli* (21.5 ± 0.86 mm) and *Bacillus cereus* (17.6 ± 0.43 mm). The petroleum ether and water extracts showed minimum and restrained antibacterial activity with the diameter ranges of 12–15 mm and 8–11 mm respectively (Sen and Batra 2012).

Table 3.1 Minimum inhibitory concentration of plant extracts in inhibiting several microorganisms (Zaidan et al. 2005)

Plant extracts	Microorganisms	Minimum inhibitory concentration (MIC, μg/disk) (mm)
Andrographis paniculata	*Staphylococcus aureus* (ATCC 25923)	1000 (6 ± 0.1)[a]
	Methicillin resistance *Staphylococcus aureus* (wild type)	250 (8 ± 0.1)[a]
	Escherichia coli (ATCC 25922)	NA
	Klebsiella pneumoniae (WHO 1995/4)	NA
	Pseudomonas aeruginosa (ATCC 27853)	2 (8 ± 0.1)[a]
Vitex negundo	*Staphylococcus aureus* (ATCC 25923)	NA
	Methicillin-resistant *Staphylococcus aureus* (wild type)	NA
	Escherichia coli (ATCC 25922)	NA
	Klebsiella pneumoniae (WHO 1995/4)	NA
	Pseudomonas aeruginosa (ATCC 27853)	NA
Morinda citrifolia	*Staphylococcus aureus* (ATCC 25923)	1,000 (7.3 ± 0.1)[a]
	Methicillin-resistant *Staphylococcus aureus* (wild type)	NA
	Escherichia coli (ATCC 25922)	NA
	Klebsiella pneumoniae (WHO 1995/4)	NA
	Pseudomonas aeruginosa (ATCC 27853)	NA
Piper sarmentosum	*Staphylococcus aureus* (ATCC 25923)	2000 (9)
	Methicillin-resistant *Staphylococcus aureus* (wild type)	1000 (8)
	Escherichia coli (ATCC 25922)	NA
	Klebsiella pneumoniae (WHO 1995/4)	NA
	Pseudomonas aeruginosa (ATCC 27853)	2000 (12)
Centella asiatica	*Staphylococcus aureus* (ATCC 25923)	1000 (5)
	Methicillin-resistant *Staphylococcus aureus* (wild type)	1000 (7)
	Escherichia coli (ATCC 25922)	NA
	Klebsiella pneumoniae (WHO 1995/4)	NA
	Pseudomonas aeruginosa (ATCC 27853)	NA

NA no activity

[a]Parentheses indicate the inhibition zone and standard deviation

Table 3.2 Inhibition concentration, IC_{50} (%v/v) values for *R. officinalis* essential oil, α-pinene, β-pinene, and 1,8-cineole (Adapted from Wang et al. 2012)

	Plant extract inhibition concentration (IC_{50})			
Cancer cell type	*R. officinalis* essential oil	α-Pinene	β-Pinene	1,8-Cineole
SK-OV-3	0.025	0.052	0.12	1.10
HO-8910	0.076	0.11	0.16	2.90
Bel-7402	0.13	0.32	0.43	3.47

Adapted from Wang et al. 2012

For antifungal activity, *Aspergillus flavus* and *Rhizopus stolonifera* were inhibited effectively by ethanol plant extract with a diameter of 21.6 ± 0.32 mm and 20.1 ± 0.62 mm respectively. Meanwhile, the methanolic extract showed proficient antifungal activity against *Fusarium oxysporum* (22.4 ± 0.86 mm) and *Aspergillus niger* (20.1 ± 0.62 mm). The smallest inhibition zone of antifungal activity was reported to be for petroleum ether and water plant extracts, with diameter ranges of 15–18 mm and 9–12 mm respectively against all pathogenic fungal strains (Sen and Batra 2012).

In addition, in the MICs of each plant extract, only ethanol and methanol showed the lowest concentration against the microorganisms examined. The lowest MIC value was reported to be for the methanol extract of *Melia azedarach* L. plant, with 22.4 μg/mL against bacteria *Bacillus cereus*, followed by ethanol extract (37.6 μg/mL) against *Pseudomonas aeruginosa.* For the inhibition of fungal strains, ethanol and methanol extracts needed 39.5 μg/mL and 47.3 μg/mL against *Rhizopus stolonifera* and *Aspergillus flavus* respectively (Sen and Batra 2012).

Throughout the overall discussion, Sen and Batra (2012) concluded that the antibacterial and antifungal effectiveness were largely dependent on the solvent type used. The organic extracts (ethanol and methanol) demonstrated more effective antibacterial and fungal activities compared with aqueous extracts. This result clearly illustrates that the presence of nonpolar residues produced by organic solvent extracts possesses higher bactericidal and bacteriostatic potential. In addition, most antibiotic constituents have been reported to exist as aromatic or saturated organic molecules, which are easily solubilized in organic solvents, especially alcohols (Cowan 1999).

3.1.4 Anticancer Activities

New approaches in controlling the malignant diseases caused by the failure of conventional chemotherapeutic drugs to cure advanced invasive carcinoma are critically needed (Faried et al. 2007). Throughout history, diverse chemical structures and biological constituents contained inside medicinal plants have been reported to have potential against several health disorders, including cancer (Plengsuriyakam

et al. 2012). Generally, by implementing the medicinal plant, the occurrence of cancer can be controlled by blocking, reversing, and slowing the development of the disease through natural anticancer constituent administration (Faried et al. 2007).

In 2012, an anticancer potential of *Rosmarinus officinalis* L. essential oil was assessed by Wang et al. (2012). The anticancer activity of the plant extract essential oil was compared with that of three of its main constituents: 1,8-cineole (27.23%), α-pinene (19.43%), and β-pinene (6.71%) against three human cancer cells; HO-8910, SK-OV-3 (ovarian cancer cells), and Bel-7402 (hepatocellular liver cancer cells). This in vitro anticancer evaluation was conducted using MTT assay and results recorded as cell viability (%), dependent on the manipulated dosage of the plant extracts.

Depicting the results, the survival of the three cancer cell lines decreased as the plant extract dosage increased. At a plant extracts dosage concentration of 0.0625%, v/v), the essential oil cell viability of 1,8-cineole, β-pinene, α-pinene, and *R. officinalis* against SK-OV-3 was 93.03%, 67.77%, 45.85%, and 36.13% respectively. At the maximum dosage (1% v/v), of plant extracts, *R. officinalis* essential oil, β-pinene, and α-pinene showed strong sensitivity against the three human cancer cells, whose cell viability was reported to be lower than 11%. Meanwhile, 1,8-cineole demonstrated poor cytotoxicity toward all cancer cell lines examined. In addition, among plant extracts, *R. officinalis* essential oil exhibited the strongest anticancer potential compared with the main constituents tested. The inhibition concentration (IC_{50}) of *R. officinalis* essential oil and three of its main components was recorded in Table 3.4.

Table 3.4 shows that SK-OV-3 exhibits greatest sensitivity toward *R. officinalis* essential oil with the lowest IC_{50} value (0.025), compared with three of its main components. Similar to the other cancer cells (HO-8910 and Bel-7402), *R. officinalis* essential oil inhibited these cancer cells efficiently with IC_{50} values of 0.076% v/v and 0.13% v/v. However, 1,8-cinoele was reported to have the lowest sensitivity against the three cancer cells examined, with IC_{50} values of 1.10%v/v (SK-OV-3), 2.90% v/v (HO-8910), and 3.47% v/v (Bel-7402). These results concluded that the successful anticancer potential showed by *R. officinalis* essential oil may have been attributable to the minor components contained, which may make a significant contribution to the pharmacological activity of the essential oil.

In another study, traditionally used Thai phytomedicines were macerated from their different plant segments by using solvent extraction techniques (ethanol, methanol, and petroleum ether). In vitro cytotoxicity of the plant extracts against several human cancer cells such as cervix cancer (HeLa) cells, multidrug resistance CEM/ADR5000 leukemia cells, and CCRF-CEM leukemia cells. Two anticancer activity evaluations were accessed: MTT assay for HeLa cells, whereas the inhibition rate of both leukemia cell types were calculated using XTT assay. Both methods used the viability (%) and inhibition concentration value (IC_{50}) recorded. The medicinal plants consisted of *Muehlenbeckia platyclada*, *Pouzolzia indica*, *Cayratia trifolia*, *Oroxylum indicum*, *Gynura pseudochina* var. *hispida*, *Gynura pseudochina*, *Basella rubra*, *Basella alba*, and *Rhinacanthus nasutus* (Siriwatanametanon et al. 2010).

Table 3.4 Particle size comparison of several essential oils-loaded nanocarriers in specific delivery systems

Essential oil	Nanocarrier	Delivery system	Range of particle size (nm)	References
Syzygium aromaticum	Liposome	–	350–380	Sebaaly et al. (2015)
Minoxidil and Rosemary	Liposome	Transdermal	180–230	Kiaee et al. (2016)
Boswellia and *Commiphora*	Solid lipid nanoparticle	Oral	100–150	
Zataria multiflora	Solid lipid nanoparticle	–	600–650	Moghimipour et al. (2012)
Eugenia caryophyllata	Solid lipid nanoparticle	Oral	400–510	Fazly Bazzaz et al. (2018)
Turmeric and Lemongrass	Polymeric nanoparticle	–	360–900	Natrajan et al. (2015)
Phytolacca decandra	Polymeric nanoparticle	Oral	100–110	
Oreganum vulgare L.	Polymeric nanoparticle	Oral	40–80	Hosseini et al. (2013)
Azadirachta indica A. Juss	Micellar nanoparticle	Intranasal	17–31	Rinaldi et al. (2017)
Eucalyptus globulus	Micellar nanoparticle	Oral	50–100	Sugumar et al. (2015)
Curcuma longa L.		Intranasal	33–38	

The HeLa cells were inhibited efficiently by petroleum ether and ethanol extracts of *Rhinacanthus nasutus*, with viability of 45% and 37% respectively. Meanwhile, the IC_{50} values of these plant extracts were 24.88 ± 0.69 (petroleum ether) and 3.63 ± 1.99, which showed the lowest inhibition concentration against human cervix cancer cells. This significant success was followed by *Oroxylum indicum* (ethanol). Both leukemia cells, CCRF-CEM and CEM/ADR5000, have been strongly inhibited by *Pouzolzia indica* (petroleum ether), with IC_{50} values of 9.75 ± 0.29 and 10.48 ± 0.12 respectively. *Rhinacanthus nasutus* (methanol) and *Gynura pseudochina* var. *hispida* (ethanol) were also highly sensitive toward the leukemia cells, which more specifically inhibited multidrug resistance CEM/ADR5000 cells at IC_{50} value of 18.72 ± 0.10 and 23.50 ± 0.12 respectively. Some plant extracts showed cytotoxic effects against all human cancer cells examined; however, some extracts only inhibited one of the cancer cells. For example, *Rhinacanthus nasutus* (ethanol) successfully inhibited both leukemia cells and HeLa cells, whereas *Pouzolzia indica* (petroleum ether) were only sensitive toward the two leukemia cells (Siriwatanametanon et al. 2010)

Thus, the study concluded that *Rhinacanthus nasutus* and *Pouzolzia indica* may contain novel natural constituents that have the potential to be produced as anticancer agents. Interestingly, the multidrug resistance of leukemia cells was reported to

be highly resistant to vinblastine, paclitaxel, doxorubicin, and other chemotherapeutic drugs (Efferth et al. 2008), but only weak-cross resistance was found to be for the present Thai medicinal plants. This study suggested that the valuable adjuncts that might be yielded by plant extracts can be implemented in standard chemotherapy in cases of drug-resistance and refractory tumors. Further investigations regarding in vivo anticancer activity of potential plant extracts and identification of their active constituents were also recommended by the researchers (Siriwatanametanon et al. 2010).

Ngamkitidechakul et al. (2010) investigated the antitumor effect of Asian traditional medicine, *Phyllanthus emblica* L. (PE) using an in vivo two-stage tumorigenesis assay. Initially, the phytomedicine has been extracted from its fruits by using aqueous solution and its major chemical constituents were determined by using the chromatography method. The constituents contained were approximately 43% tannins, 21% gallic acid, and 11% uronic acid.

Through in vivo evaluation of the antitumor effect, the 7,12-dimethylbenz(a) anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA) solution induced tumorigenesis on I skin of mice. This tumorigenesis induction included two stages where the whole group of mice is initially treated with single topical administration of 290 nmol of DMBA on their shaven backs as a tumour initiator. After 1 week, the TPA was topically administered on treated skin twice weekly for 20 weeks as a tumour promoter. For the plant extract group, the animals were treated with topically applied PE fruit extract (1, 2, and 4 mg), 1 h after topical administration of TPA until the end of the experiment. The presence of benignity on the treated skin area was counted and measured using a vernier caliper. The results were expressed as the average number of tumors per mouse (multiplicity) and percentage of tumor-bearing mice (incidence). The findings were compared with those of a control animal group (treated with DMBA/TPA only).

The animal groups treated by plant extracts, DMBA and TPA, showed more than 50% reduction in tumor numbers and volumes over a 20-week period. The control group demonstrated that the incidence of benignity reached 100% within 10 weeks of tumor promotion, with the average number of tumors per mouse at 10 and 20 weeks being 7.7 and 11.8 respectively. Additionally, the tumor volumes of the control group were reported to have increased from 3.4 mm^3 (week 10) to 8.1 mm^3 (week 20). In addition, there was a rare finding regarding the two high-dose animal groups (PE at 2 and 4 mg), which were revealed to produce fewer tumors compared with the control group and the group receiving 1 mg of PE.

Therefore, the researchers concluded that the aqueous extract of PE had the potential to inhibit tumor generation and become a novel chemotherapeutic agent. In addition, Ngamkitidechakul et al. (2010) stated that the richness of antioxidant component, tannins, contained inside the plant extract promoted good inhibition of hydrogen peroxide production, ornithine decarboxylase activity, and DNA synthesis promoted by TPA skin tumor induction.

3.1.5 Anti-oxidant Activities

The recent numerous pieces of evidence reveal the normal process of aging, which, among the main factors of degenerative disease development, is caused by oxidative stress (Sen et al. 2010; Shirwaikar et al. 2011). In a living system, the normal physiological and biochemical processes are generated by reactive oxygen species (ROS) and reactive nitrogen species, which are firmly regulated by antioxidant enzymatic and non-enzymatic mechanisms. Low or moderate concentration of ROS/RON promote energy generation, the regulation of cell growth, phagocytosis, and production of biologically important compound synthesis. However, the overproduction of reactive species or known free radicals can lead to oxidative stress. Free radicals can affect the living cells by causing biomolecule oxidation, which leads to protein oxidation, lipid peroxidation, and DNA damage. These can lead to cell death, tissue damage, and mutagenic changes (Bhagat et al. 2011).

Recent findings show the potential of phytomedicines to promote natural antioxidant activity including flavonoids and phenolic compounds, which were found to have a successful therapeutic effect against various diseases caused by oxidative stress. Huge attention has been received by natural antioxidants of medicinal plant in recent years and there has been a great potential worldwide in the identification and implementation of the natural antioxidant agents of medicinal plants, which can have a wide scope in preventing free radicals that cause diseases in biological systems.

Sen et al. (2013) evaluated the antioxidant performance of Indian phytomedicine, known as *Meyna spinosa* Roxb. Solvents, such as methanol, ethyl acetate, and petroleum ether, were used to extract the leaves of medicinal plants. The in vitro antioxidant assays that have been applied are 2,2-diphenyl-picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging, superoxide anion scavenging, hydrogen peroxide scavenging, and nitric oxide radical scavenging. In addition, the total amounts of polyphenolics and flavonoids contained in *Meyna spinosa* were determined.

Methanol extract was reported to have the highest polyphenolic content and demonstrated better antioxidant potential, compared with the other two methods. Total amounts of flavonoids and phenolics contained inside *Meyna spinosa* methanol extract were (58.50 ± 0.09) mg quercetin equivalents/g and (90.08 ± 0.44) mg gallic acid equivalents/g respectively. In addition, the antioxidant performance of each in vitro assay showed the IC_{50} of (16.4 ± 0.41), (24.1 ± 0.33), (35.9 ± 0.19), (126.8 ± 2.92), and (23.7 ± 0.09) demonstrated by DPPH, hydroxyl radical, superoxide anion, hydrogen peroxide scavenging activity, and nitric oxide radical assays. These antioxidant potentials of plant methanol extract were revealed to be similar to the standards (Sen et al. 2013).

Thus, Sen et al. (2013) stated that there was a direct relationship between the total phenolic content and the antioxidant performance of plant methanol extract, which may be the major contributors to the antioxidant activity of the

phytomedicine. The methanol extract of *Meyna spinosa* is reported to be confirmed as a potential source of natural antioxidant (Sen et al. 2013).

At the end of the twentieth century, an investigation into the antioxidant properties of *Nigella sativa* L. essential oil, was evaluated using thin layer chromatography (TLC) screening and DPPH assay methods. The plant material segment was chosen from the seeds and extracted using the Soxhlet extraction technique. According to gas chromatography-mass spectrometry (GC-MS) analysis, the extracted aromatic plant seeds were found to consist of a mixture of the monoterpenes group, such as the major components thymoquinone (30–48%), p-Cymene (7–15%), carvacrol (6–12%), and the minor components 4-terpineol (2–7%), anethole (1–4%), and sesquiterpene longifolene (1–8%) (Burits and Bucar 2000).

Based on the TLC screening method, DPPH reagent was used to spray on the eluted essential oil with the aim of evaluating its antioxidant activity. With the DPPH reagent, the active components with antioxidant activity were detected as a yellow spot on a violet background. Those constituents of essential oils that turned from purple to yellow spots within the first 30 min (after spraying) were considered for further antioxidant analysis. As a result, two spots appeared immediately after spraying the chromatogram, which were from constituents of thymoquinone and carvacrol. The two other zones from the components anethole and 4-terpinol changed their color 15 min later.

Next, the DPPH analysis conducted to determine the ability of these four components and the pure essential oil to act as a donor for hydrogen atom or electrons was spectrophotometrically measured. The antioxidant activity of this medicinal plant is shown as Table 3.3.

In another investigation, a Malaysian common phytomedicine, *Eurycoma longifolia*, alternatively known as Tongkat Ali, was evaluated for its antioxidant activity by using an in vitro DPPH assay. This medicinal herb was extracted using a simple maceration technique. Its antioxidant evaluation was accessed dependent on the manipulated dosage concentration of plant extract (10, 25, 50, 100, and 250) μg/mL.

Depicting the result, the antioxidant performance of plant extract correlated directly with increased concentration. The lowest concentration of *Eurycoma longifolia* (10 μg/mL) demonstrated the lowest inhibition of scavenging activity (20.39 ± 0.186), whereas the highest dosage concentration (250 μg/mL) exhibited the greatest scavenging activity inhibition (61.132 ± 0.113).

3.2 Challenges of Phytomedicine Product Development

Phytomedicines are commonly categorized as secondary plant metabolites, whereby these metabolites are being chemically isolated and identified. Challenges are expected to occur during phytomedicine screening and their active constituent extraction process (Qureshi and Al-Bedah 2013). Secondary metabolites of phytomedicines are usually found under very low conditions and the active constituents are varied, depending on several factors such as the anatomy of the plant segments (flower, seed, leaf, root, and others) and also type of ground, humidity, storage, and geographical area (Egert and Rimbach 2011).

Table 3.3 Antioxidant activity of *Nigella sativa* essential oil and seed extraction of its main constituents

Test components	IC_{50}
Essential oil	460.0
Thymoquinone	211.0
Carvacrol	28.8
Quercetin	1.31
Butylhydroxytoluene	12.12
Ascorbic acid	3.76

Adapted from Burits and Bucar 2000

The high-throughput screening (HTS) technique is assessed for drug component screening, normally in pharmaceutical industries. Some researchers have tried this method of screen the constituents from medicinal plants; however, it was reported that this method is not suitable for phytomedicines. In addition, some of these medicinal plants revealed false information during HTS (Kingston 2011).

Numerous in vitro findings for plant extracts have reported to have surprising potential for pharmacological activities of phytomedicines, However, for in vivo studies, the significant biological potential of these medicinal plants are fewer or non-effective findings, attributable to their poor solubility, instability, and resulted in low bioavailability and therapeutic potential (Gunasekaran et al. 2014).

3.3 Nanotechnological Approach in Enhancing the Pharmacological Bioavailability of Phytomedicines

Nanotechnology can be an efficient tool in intensifying the bioavailability and therapeutic potential of the bioactivity of phytomedicines; in addition, all the limitations of these plant extracts can be eradicated. The aqueous solubility and permeability of phytomedicines through a biological membrane can be augmented by reducing the size of phytomedicines by encapsulating them in nanocarriers.

Liposomes, niosomes, solid lipid nanoparticles, and micelles are common novel drug delivery systems with the ability to deliver herbal drug constituents efficiently. By incorporating phytomedicines into these nanocarriers, plant extract solubility and stability can be enhanced, toxicity reduced, pharmacological activity intensified, drug delivery sustained, and the constituent protected from any physical and chemical degradation (Bilia et al. 2014).

As discussed, the significant potential of incorporating phytomedicine into nanotechnology is attributable to the ability of the nanocarriers to reduce the size of the plant extracts and modification of surface properties. Hence, the next sub-sections discuss these two beneficial factors of nano-phytomedicine.

3.3.1 Phytomedicine Particle Size Reduction

Oral administration is a favorable route for delivering phytomedicines, achieving patient compliance and manufacturing advantages. Through the absorption phase of orally administered drugs, there are two critical slower rate-determining steps (RDSs). These steps include the drug dissolution and permeation rate through the membrane. Hydrophobic or poor solubility of drug dissolution is considered as the RDS and the absorption of such drugs is known as the limited drug dissolution rate (Gunasekaran et al. 2014).

Enhancing drug hydrophilicity with high aqueous solubility leads to a rapid drug dissolution rate and resulted in a higher permeation rate of the drug through the biological membrane. By reducing the size of phytomedicines, various routes of well-formulated nano-phytomedicines can be enhanced in terms of absorption, dissolution, bioavailability, and therapeutic efficacy of the drug; in addition, it can be implemented at lower dosages (Gunasekaran et al. 2014).

Transdermal administration of medicinal plants known as *Artemisia arborens* has been enhanced by incorporating it with solid lipid nanoparticles, a novel drug delivery system. Lai et al. (2006) evaluated the skin permeation of *Artemisia arborens* essential oil-loaded solid lipid nanoparticles. Two SLN formulations were developed by using two different surfactant types; Poloxamer 188 (SLN 1) and Miranol Ultra C32 (SLN 2), and Compritol 888 ATO as lipid components by using a high-pressure homogenization technique. The average lipid particle size of the formulations was monitored for 2 years to examine its stability. As a result, a day after the nanocarrier formulations, the SLN 1 was reported to have a size of 223 nm at a polydispersity index (PDI) of 0.243. Meanwhile, SLN 2 formulation had a size of nanoparticles of 219 nm at a PDI of 0.301. After 2 years of storage, the particle size of the SLN 1 and SLN 2 formulations were slightly increased to 242 nm (0.285 PDI) and 239 (0.301 PDI) respectively.

Depicting the the bioactivity result, the capability of solid lipid nanoparticles greatly enhanced the oil accumulation on pig skin, whereas the control solution showed a high oil permeation rate due to the successful penetrating, moisturising, and restructuring characteristics of *Artemisia arborens* oil. These matters are related to the smaller plant extract particle size reported (223 nm).

Su et al. (2008) evaluated the pharmacological potential of *Radix salvia* medicinal plant enriched with nanoparticles by using a spray drying method and this nano-phytomedicine was investigated for its potential to treat diseases such as angina pectoris, coronary heart disease, and myocardial infarction. The results showed the improved bioavailability of the plant extract due to the particle being reduced after being incorporated into nanoparticles.

3.3.2 Phytomedicine Surface Properties Modification

Various techniques can be implemented in modifying the surface properties of phytomedicine, by surface coating with stabilizing, hydrophilic, and mucoadhesive polymer or surfactant, leading to changes in hydrophobicity, mucoadhesiveness, stability properties, zeta potential of nanoparticles, and protein adsorption on their surface (Gunasekaran et al. 2014).

Commonly among oral administration of drugs, the gastrointestinal tract is frequently involved and resulted into reduced drug retention time in the blood circulation and leading to limitation of the drug bioavailability. Incorporation of phytomedicines into nanocarriers can be one possible way of enhancing the absorptive properties of herbal drugs and increasing its retention time at a mucosal and epithelial level (Gunasekaran et al. 2014).

In addition, the ions produced on phytomedicine-incorporated nanoparticles resulting in the modification of surface properties can exhibit a reduction in the particle size of herbal drugs. Non-ionic surfactant is usually related to producing ions during dispersion through aqueous solution, where this is attributable to the presence of H_3O^+ and OH^- ions. The negative charge will develop if the nanoemulsion system is under acidic condition (pH 3–6), whereas the positive ion will be observed for natural conditions of the nanoemulsion system (pH more than 7) (Rai et al. 2018).

The mechanism involves a higher degree of surface charge on surfactant aggregation through aqueous medium, which able to keep away between the nanoparticles owing to the presence of a strong intermolecular repulsive force (Rai et al. 2018). Monosurfactant-based nanoemulsions are stabilized by this repulsion force among charges and promote all the charges of nanodroplets in Brownian motion (Vilasau et al. 2011). Figure 3.1 shows the droplets in motion approach together with the stronger repulsive force among charges, encouraging them to finally move away after an elastic collision through the oil in water nanoemulsion system.

This repulsive force between two nano droplets enables any gravitational separation, flocculation, and coalescence phenomena to be avoided and facilitates the formation of smaller nanoparticle sizes. The nanoemulsion system has been widely incorporated into medicinal plants owing to its ability to develop extreme particle sizes of nano-phytomedicines.

3.3.3 Phytomedicine-Loaded Nanocarriers

Incorporation of medicinal plant into nanocarriers is one approach to representing feasible and efficient herbal drug delivery, avoiding bioactive constituents interacting with the biological environment, enhancing the stability of phytomedicine components, reducing volatility and toxicity, and hence resulting in high efficacy, bioavailability, and therapeutic effects (Bilia et al. 2014).

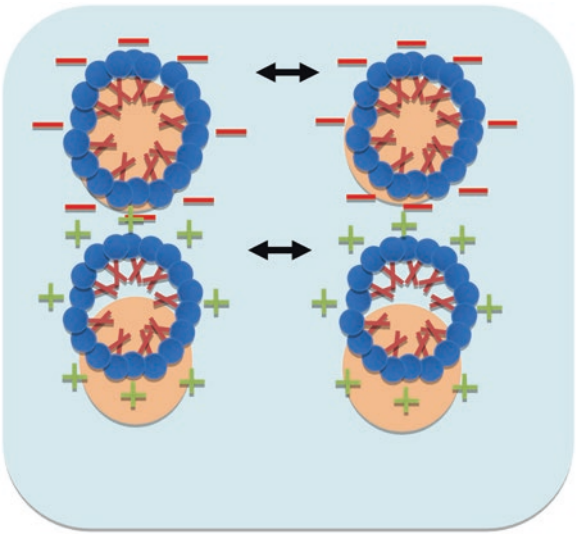

Fig. 3.1 Stability mechanism between nanoparticle droplets in the nanoemulsion system (Adapted from Rai et al. 2018)

Through incorporation of phytomedicine into nanocarriers, numerous desirable features of drug therapy can be achieved: (1) deep tissue penetration owing to nanometric size; (2) sustained and controlled drug release; (3) protection of therapeutic cargo at both extracellular and intracellular levels; (iv) subcellular trafficking and cellular uptake (Bilia et al. 2014).

The different nanocarriers used can be developed via different nano-phytomedicine formulation methods, which include high-pressure homogenizer, ultrasonication, complex coacervation, co-precipitation, nanoprecipitation, supercritical fluid, phase inversion, and self-emulsification methods. Several types of nanocarriers are commonly being incorporated into phytomedicine such as liposomes, niosomes, solid lipid nanoparticles, and micellar nanoparticles. Hence, the next sub-sections discuss each recent nano-phytomedicine investigation.

3.3.3.1 Liposomes

In the early 1970s, liposomes were developed and implemented as the first nanocarriers introduced into novel drug delivery systems and have been widely discussed among researchers in colloidal delivery fields (Bilia et al. 2014). It contains one or more phospholipids, which are molecules with head and tail groups. In aqueous medium, the lipids are separated, whereas the hydrophilic drugs are encapsulated in an aqueous compartment, and the membranes are incorporated into hydrophobic substances.

Size, the number of lamellae, and surface charge are three factors involved in the characterization of liposomes. As for surface charge, liposomes can be classified into anionic, cationic, and neutral. Regarding their size and number of lamellae,

they can be classified as oligo-, uni- or multilamellar, and small, large, or giant respectively. Liposomes in a unilamellar pattern only contain of single bilayer and consist of various size ranges, which are small unilamellar (25–100) nm, large unilamellar (100 nm to 1 μm), and giant unilamellar with diameters of more than 1 μm. Other than that, multilamellar liposomes contain several concentric lamellae, which are usually found in more concentrated systems (Bonifácio et al. 2014).

The potential of liposomes in novel drug delivery systems to enhance the therapeutics of pharmacological activities among essential oils have been widely discussed (Bonifácio et al. 2014). Two phytomedicines, known as *Eucalyptus camaldulensis* and *Ligusticum chuanxiong*, were investigated for their transdermal administration in curing dermatological diseases (Moghimipour et al. 2012; Zhang et al. 2012).

A preliminary study on *Eucalyptus camaldulensis*-loaded liposomes as antifungal agents for dermatophyte growth has been evaluated by Moghimipour et al. (2012). Liposomal gel of *Eucalyptus* was developed and its rheological properties studied. Depicting the determination of the main constituents of essential oil, limonene, 1,8-cineole, phenol, pinene, and terpinen was found and analyzed by gas chromatography-mass spectrometer technique. The particle size of the medicinal plant liposomal formulation was reported to be within a range of 40.5 and 298 nm, whereas the formulated *Eucalyptus*-loaded liposomal gel was reported to improve antifungal activity of *Eucalyptus camaldulensis* essential oil by the rheological properties, resulting in no significant thixotropy being observed.

Zhang et al. (2012) reported the successful performance of transdermal administration of the medicinal plant known as *Ligusticum chuanxiong* Hort., isolated from its rhizomes, to reduce hypertrophic scarring in a rabbit ear model. Hypertrophic scarring is produced on top of dermal skin owing to excessive fibrolast production and collagen diposition, especially during wound-healing of skin conditions.

Previously, *Ligusticum chuanxion*- loaded liposome (LEO) treatment promoted significantly reduced hypertrophic scarring on rabbit ears by reducing levels of collagen I and III, which correlated directly with increased *Ligusticum* oil concentration. Meanwhile, the scar elevation index (SEI) was reported to be significantly reduced with the elevation of phytomedicine composition. Therefore, these results indicate that liposomes of *Ligusticum chuanxiong* exhibit successful therapeutic effects on hypertropic scars forming in the rabbit ear model and may have the potential to cure hypertropic scars in humans (Zhang et al. 2012).

3.3.3.2 Niosomes

Niosomes were first developed and formulated in novel drug delivery areas during the mid-1970s by researchers in cosmetic industries. Niosomes contain the vesicles of non-ionic surfactants and form the lamellar structure by combining the alkyl or dialkyl polyglycerol ether class of the surfactant with cholestrol as an excipient. The closed bilayer vesicle of a non-ionic surfactant is formed in aqueous media owing to its amphiphilic nature by some form of energy such as physical agitation and

heating to form the lamellar structure. Through the structure, hydrophobic segments of surfactant are attracted to each other and oriented away from aqueous medium, whereas the hydrophilic segments interact further with aqueous solvent (Kazi et al. 2010).

In addition, niosomes are known to be alternatives to liposomes that possess a better stable structure with no specific condition during storage and are chemically stable. This approach introduce a low-cost production of niosomes into novel drug delivery systems. Fabrication of niosomes has been implemented into numerous therapeutic moieties whether among hydrophilic, lipophilic, as well as amphiphilic drugs, which were reported to enhance the oral and topical bioavailability of the drugs by providing them with protection from the biological environment, resulting into improvements in therapeutic performance (Debnath and Kumar 2015).

Through the anti-inflammatory performance of niosomal *Zingiber cassumunar* (ZC) gel, the result was compared with blank niosome gel (negative control), and two positive controls: piroxicam gel and hydrocortisone cream. Ear edema on the right ear of mice underwent application of 0.075 mg of croton oil (diluted in acetone), whereas the left ear had acetone alone administered. After the acetone had evaporated, in the same area, the ear underwent topical application of each treatment (0.1 g/each). The ear thickness was calculated by using a digital vernier caliper to determine the ear edema inhibition rate between treated and non-treated areas (Priprem et al. 2016).

According to the result, there was no significant difference in the percentage of ear edema inhibition among topical application of ZC niosomal gel, piroxicam gel, or hydrocortisone cream ($p < 0.05$). The ZC niosomal gel had a maximum inhibition rate of ear edema of 75% at 3 h after topical administration and was slightly decreased. Meanwhile, neither blank niosomal gel nor blank gel showed promotion of anti-inflammatory activity. Depicting the discussion of this previous study, the component D contained inside the phytomedicine possesses a successful anti-inflammatory activity and although the encapsulation of *Zingiber cussumunar* promoted immediate permeation, this easily localized the component D on the target area (Priprem et al. 2016).

Another previous piece of research assessed the anti-inflammatory potential of the aromatic medicinal plant, *Zingiber cussumunar* essential oil, also known as Plai oil, encapsulated with niosome gel. Pure essential oil from the Plai rhizome was macerated by using a steam distillation extraction technique, and its niosomal gel was developed using the chloroform film method. Lipopolysaccharide (LPS)-induced subcutaneous inflammatory assay was injected into the subcutaneous area in rats to evaluate the anti-inflammatory performance of three gel types; non-oil-encapsulated niosome gel (blank), essential oil-encapsulated niosome gel, and neurofen gel (positive control), topically administered on an inflamed part of Wistar rats via ultrasound. Approximately 400 g of each gel was used for the treatment and all treatments were repeated daily for 3 days (Leelarungrayub et al. 2017).

The skin temperature and blood flow (flux/min) of the inflammed area were determined using an optical probe of a Doppler blood flow meter. As a result, a significant increase in the skin temperature and blood flow of the rats was shown

after LPS injection on days 1 and 2. However, topical administration of the three gels resulted in reduced skin temperature and blood flow of the inflamed part on days 2 and 4. Gel formulation with Plai oil niosome showed the lowest values of rats' skin temperature and blood flow, and this indicated better topical anti-inflammatory performance of *Zingiber cussumunar* essential oil after fabrication with niosomes (Leelarungrayub et al. 2017).

3.3.3.3 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) represent the nano-sized scale of a nanocarrier formulated using lipids that remain solid at room temperature (and/or body temperature). This nanocarrier is broadly implemented to be encapsulated with a lipophilic drugs component and become beneficial in terms of stability over liposomes and niosomes. The lipid components consist of wide range of lipids and lipid-like molecules such as waxes or triacylglycerol that the lipid particle can achieve within a range size of between 50 nm and 1 μm. Non-ionic surfactants are used to stabilize the solid lipid particles and the active constituents are solubilized homogeneously whether in the solid core or on the outside (Bilia et al. 2014).

A solid particle structure possessing the enhancement of the solubility and bioavailability of pharmacologically active elements, leading to incremental chemical protection, sustained drug release, and increased encapsulation efficiency. These promoted solid lipid nanoparticles have become advantageous in novel drug delivery systems (Bilia et al. 2014).

Lai et al. (2006) evaluated the skin permeation of *Artemisia arborescens*-loaded solid lipid nanoparticles. Two SLN formulations were developed using two different surfactant types, Poloxamer 188 (SLN 1) and Miranol Ultra C32 (SLN 2), as well as Compritol 888 ATO as a lipid component by using a high-pressure homogenization technique.

The average lipid particle size of the formulations was monitored for 2 years to examine its stability. As the results, a day after the nanocarrier formulations, the SLN 1 was reported to have a size of 223 nm at a PDI of 0.243. Meanwhile, the SLN 2 formulation had a nanoparticle size of 219 nm at a PDI of 0.301. After 2 years of storage, the particle size of the SLN 1 and SLN 2 formulations was slightly increased to 242 nm (0.285 PDI) and 239 (0.301 PDI) respectively.

In addition, the permeation and accumulated rates of formulated SLN-loaded *Artemisia arborescens* phytomedicine (SLN 1 and SLN 2) were evaluated using an in vitro diffusion method through newborn pig skin and the results were compared with those of almond oil as a control. According to the finding, the capability of solid lipid nanoparticles greatly enhanced the oil accumulation on the pig skin, whereas the control solution showed a high oil permeation rate owing to the successful penetrating, moisturising, and restructuring characteristics of almond oil (Lai et al. 2006).

3.3.3.4 Micellar Nanoparticles

Micellar nanoparticles were invented during the mid-1990s by scientists at Novavax. This technology is the latest nanocarrier to be developed and patented, which was implemented and subsequently rolled out as the first nano-engineered transdermal lotion known as Estrasorb™ in 2003. Estrasorb™ is a nanoemulsion-based lotion topically used as estrogen modification for the human system and widely manufactured on a kiloton scale. All ingredients used in this lotion were reported to be generally recognized as safe (Lee et al. 2010).

It is a nanotechnology-based formulation that reported to be a successful nanocarrier that can accommodate a wide range of therapeutic drugs, especially for lipophilic components that have various physicochemical properties. The technology offers drugs with high concentrations to penetrated into the skin and functionally create a drug depot on the stratum corneum and epidermis. In addition, it is cosmetically more convenient and acceptable to several patients (Lee et al. 2010).

Generally, micellar nanoparticles are developed by the formation of self-aggregated amphiphilic molecules (emulsifier or surfactant) to form micelles. Micelles are formed in aqueous solution where the polar segment of an amphiphilic molecule faces the outside surface, whereas the nonpolar region forms the core of the micelles. Micelles enable delivery of hydrophobic or lipohilic active constituents by entrapping them inside the core, leading to sustained and controlled drug release, enhanced physical and chemical stability of entrapped constituents, reduced toxicity, improved drug pharmacokinetics, and resulted in high bioavailability and great therapeutic effects. Micelles are frequently spherical or rounded in shape within extremely tiny particles ranging in size between 10 and 100 nm, dependingt on the system compositions. Several typical techniques are used to form micellar nanoparticles, such as solvent evaporation, the dialysis method, solid dispersion, and oil in water emulsion. Of these, emulsion is the most emerging micelle preparation method that is broadly discussed and implemented, besides being suitable and convenient for transdermal application (Joseph et al. 2017).

Previously, in 2015, transdermal micelles of *Foeniculum vulgare* Mill. in nanoemulsion systems were formulated and their in vivo antidiabetic activitiy was evaluated. Streptozotocin (STZ)-induced diabetes (60 mg/kg) was intraperitoneally injected into rats fasted overnight and these animals were considered to be diabetic rats. Thirty-six rats were divided equally into six groups, where the first group was considered to be the normal healthy control group (group I). The diabetic rats were divided into another five groups, of which group II was a diabetic control of rats with no treatment, groups III to V consisted of the animals treated topically by nanoemulsions of *F. vulgare* (F1, F2, and F3) at a dose of 30 mg/kg.

The last group of animals (group VI) were treated by transdermal administration of pure *F. vulgare* essential oil at 30 mg/kg. All treatments were transdermally administered into the rat dorsal area at 6 cm^2 using a gentle rubbing method. One hour after treatment administration, all animals consumed oral glucose at a dosage of 1 g/kg of body weight to determine the glucose tolerance of each tested

formulation. Animals' blood samples were collected 1 h before medication application and 0.5, 1, 2, and 4 h after oral glucose administration for determination of blood plasma glucose (Mostafa et al. 2015). Depicting the result, the micelles of *F. vulgare* in the nanoemulsion system were successfully formed at a particle size range of 44–105 nm with encapsulation efficiency reaching 64%. In addition, transdermal administration of *F. vulgare* and *F. vulgare* essential oil (FEO) micelle formulations showed a significant reduction of plasma glucose level among diabetic rats 0.5, 1, 2, and 4 h after oral glucose administration, hence demonstrating a short-term effect on antidiabetic activity.

After 4-h administration, the animals treated with pure *F. vulgare* indicated a plasma glucose level of 296.8 ± 5.658, whereas nanoemulsion formulations (F1, F2, and F3) promoted rat plasma glucose levels of 277.4 ± 8.023, 290.0 ± 8.534, and 288.2 ± 9.595 respectively. The untreated diabetic rats demonstrated a plasma glucose level of 316.7 ± 6.145. Furthermore, there was no significant difference in antidiabetic potential up to 4 h after oral glucose administration among pure FEO and three formulated FEO nanoemulsions (Mostafa et al. 2015).

Additionally, as F1 was reported to have the best antidiabetic effect, it was then tested for two extra doses for 60 and 120 mg/kg. The result demonstrated a significant decrease in plasma glucose level of F1 compared with FEO at the same doses 7 days after administration. Hence, this study revealed that the micellar nanoparticle of *F. vulgare* essential oil formed through the nanoemulsion system enables its antidiabetic activity to be prolonger and enhanced via transdermal administration (Mostafa et al. 2015).

On the other hand, antidiabetic and hepatoprotective potentials of cumin essential oil (CEO) micelles through the nanoemulsion system has been investigated by Mostafa et al. 2015. The in vivo antidiabetic and hepatoprotective activities of cumin essential oil were evaluated using oxidative stress induction in rats. Through the evaluation, 48 rats including albino species were employed and categorized into seven groups.

Initially, all animals were grouped into: (1) group 1 containing six rats was considered to be the normal healthy control group; (2) group 2 contained seven rats as a control group with single oxidative stress induced; (3) groups 3–7 consisted of seven rats each that underwent trandermal administration of 60 mg/kg of CEO nanoemulsion formulations (S2, S3, S5, S6) and pure CEO with Tween 20. On the first day, these five treatments (groups 3–7) were topically applied onto the dorsal area of the rats using the gentle rubbing method. On the second day groups 2–7 were given paracetamol orally (1 g/kg of body weight) for oxidative stress induction (Mostafa et al. 2015).

The main findings of this study were the determination of plasma total antioxidant capacity (TAC) by detected plasma malondialdehyde (MDA) as an indicator of lipid peroxidation and oxidative stress for CEO antidiabetic activity potential. Meanwhile, the hepatoprotective properties were perceived by determination of plasma activity of aspartate transaminase (AST) and alanine transaminase (ALT) as indicators of liver function. These results were accessed by analyzing the animals'

blood samples that were taken on the 3rd, 5th, and 7th days of the experiment (Mostafa et al. 2015).

According to the in vivo antioxidant performance of transdermal CEO nanoemulsion, the S5 formulation manifests a significant increase in TAC level from the 3rd to the 7th days of the experiment, reaching a maximum value at 7 days with a nonsignificant difference ($P > 0.05$) from S3, which demonstrated the highest TAC value. Formulation S5 is reported to be better than S3 owing to the inconsistency of TAC values achieved by S3, whereas S5 increased constantly with time until reaching maximum efficacy at a 7-day interval. Based on these findings, the overall plasma TAC and MDA values of each experimental group revealed that the cumin essential oil-loaded nanoemulsion system significantly increased and decreased TAC and MDA levels respectively in rat plasma compared with controls (CEO per se Tween 20) formula, especially during the 7-day time interval of the investigation. In addition, the hepatoprotective potential of transdermal CEO nanoemulsion formulations demonstrated significant decreases in plasma AST and ALT compared with the animals topically treated with oxidative stress (group 2) and CEO per se (group 7). The S5 formula led to a good reduction in plasma AST, which was reported to be a nonsignificant difference ($P > 0.05$) from those formulations exhibiting the lowest value of AST plasma (S2) and a significant difference ($P < 0.05$) from group 2. Furthermore, the S6 formulation demonstrated the highest reduction of plasma ALT with a nonsignificant difference ($P > 0.05$) from group 1 (Mostafa et al. 2015).

Therefore, this sub-section demonstrates the successful delivery system of each of the nanocarriers loaded with phytomedicines. Transdermal administration has become the favourable topic that has been discussed through this section. Thus, Table 3.4 shows the particle size comparison of several essential oils-loaded nanocarriers in specific delivery systems. This comparison shows several of the latest investigations regarding nanocarriers in specific delivery system, which reported that the essential oils-loaded micellar nanoparticles possess the smallest particle size (each delivery system) compared with other nanocarriers. Hence, it can be suggested that the significant potential of micellar nanoparticles to carry effectively wide ranges of biological constituents of essential oils in drug delivery systems.

3.4 Conclusion

Phytomedicines were reported to possess a significant therapeutic potential within their certain pharmacological activity. However, the poor solubility and bioavailability of phytomedicine, which limit their pre- and clinical assays, encourage it to be explored using nanotechnology. Incorporation of phytomedicine into nanotechnology leads to increased solubility, absorption rate, permeation membrane, and bioavailability of the biological constituents of plants by modification of the particle surface, reducing the size of particles in phytomedicine, enriching the

phytomedicine with several nanocarriers. The nanomaterials being implemented have had significant success in magnifying the in vivo pharmacological activities of phytomedicine in several fields.

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Chapter 4
Nanoparticles and Their Application in Folklore Medicine as Promising Biotherapeutics

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Abstract Folklore medicines have been used to treat numerous diseases and ailments since ancient times and have been recognized for their better remedial value due to fewer side effects compared to allopathic medicines. Folkloric medicines generally include decoctions and mixtures made from the extracts of parts of medicinal plants to make these herbal drugs. Herbal drugs have been recently getting more attention because of their potential to treat almost all diseases. However, there are a few drawbacks such as poor solubility, poor bioavailability, low oral absorption, and instability which have limited their use. In order to overcome these problems, nanoparticles have come into play. Nanotechnology and herbal science are integrated to overcome the limitations of using herbal drugs in a scientific way, for the development of novel drug delivery system for herbal drugs with a nano dose which aids in increasing the biosolubility and bioavailability, protection from toxicity, and persistent delivery. These nanosized drug delivery systems have predetermined rates and site precise action. In folklore medicine, nanoparticles containing bioactive therapeutic agents have acquired a great deal of implication due to their impending site-specific action and use in drug delivery. Metallic nanoparticles can also be synthesized from plants due to the inherent ability of plants to accumulate metal ions. A number of plant sources recognized for their use in traditional medicine are being used to synthesize therapeutic nanoparticles. The current review aims to summarize the application of nanotechnology in herbal medicine and the use of nanoparticles synthesized from plants as promising biotherapeutic agents in folklore medicine and presents an overview of the aspects of folkloric medicine in India.

Keywords Folklore medicine · Herbal medicine · Metallic nanoparticles · Nanoparticles · Nanomedicine · Nanotechnology · Phytomedicine

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_4

4.1 Introduction

Folk medicine alludes to those conventional therapeutic practices that do not form a part of formal medical training and normally involve the use of plant-extracted medication, honed specifically by one socio-geographical population. This knowledge, although not documented, is propagated verbally by our ancestors since many generations. The use of these medicines is generally confined to a particular geographical region or group of people such as a tribe or caste community. Primeval folk practitioners accomplished their expertise by observation, examination, and imitation. A number of communities belonging to rural/native/cultural societies depend on folk medicine which has a pivotal role in the remedy of illness. The vast majority of people from developing and underdeveloped countries, who do not have access to contemporary medicine, depend on the traditional/native herbal medicine for their healthcare in spite of great advancements in contemporary medicine.

Folklore medicines have been extensively used since ancient times and are recognized by general practitioners and patients for their better remedial value as they have negligible undesirable effects in correlation to allopathic medicines (Goyal et al. 2011; Thakur et al. 2011). Local communities in developing countries have depended on the exploitation of folkloric plant-derived bioactive secondary metabolite extracts for the treatment of numerous diseases and ailments. Usage of herbs and mineral products along with "enchantment" was the foundation for early folklore medication. The primeval sources like the Atharvaveda indicated the usage of plants and herbs as healing medication which dates back to the 1200 BC, subsequently by the Kahun in Egypt (Petrie collection) in 1880 BC and the Avesta from Persia (around the sixth century AD).

Simple medicinal herbs, porridges, broths, and filtrations were integrated into folk medicine for an effective medication. For example, folk medicines, viz., *Psidium guajava* leaf extract, *Azadirachta indica* (neem) leaf extract, *Ocimum sanctum* boiled with tea leaves, *Momordica charantia* extract, and *Carica papaya* leaf extract, were being used for curing numerous diseases. Folk medicine is generally practiced in secluded areas, and several folk medications are of surprisingly high curative significance. A huge percentage of inhabitants in a number of developing countries still rely on conventional practicians, including midwives, herbalists, and bone healers who are dependent on indigenous medicinal plants to gratify their primary healthcare needs.

In South Asia, despite the accessibility of a large number of practitioners with dissimilar specializations, millions of common people still rely on self-medication using indigenous remedies based on medicinal plants. In south Asian provinces, numerous indigenous plant species are used in these preparations. Many pharmaceutical companies rely on the scientific information about tribal and folk practices which assist in identifying potential lead molecules for future drug preparation, while the pharmaceutical medicines are derived from plants, almost 75% of these were discovered by screening these potential plants in folklore medicine.

In India, the use of plant extracts has been a practice since ancient times which plays a crucial role in the well-being of communities. In the Vedas, knowledge about herbal or plant extract-based treatment and their effectiveness was conveyed by word of mouth from one generation to another. In traditional medicines, for primary healthcare, plant extracts are used by nearly 80% of the world population (Sandhya et al. 2006). However, for principal healthcare, roughly 70% of rural populations are reliant on traditional medicine such as Ayurveda, Unani, Siddha, Amchi, and folk medicine (Pandey et al. 2013).

The prehistoric and primordial science of Ayurveda has an in-depth knowledge of the medicinal plants in Indian medical systems. The effectual treatment has always been endowed by ancient knowledge combined with scientific knowledge to combat and eliminate certain diseases. Folkloric knowledge of the uses of medicinal plants has provided many important drug preparations. The medical utilities of several of these plants have been documented; however, there are many more, whose potentials are yet to be explored.

As reported by the Anthropological Survey of India, the majority of India's populations consisting of more than 4635 tribes which are spread throughout the country living near the jungles, in rural areas, and in villages depend on folkloric medicine for their primary healthcare. Because India has a varied religious, cultural, and multilingual group of people, numerous medicinal systems have been developed. The development of folklore medicine is hindered by the advancement of modern allopathic medicine and facilities.

India is a domicile for a large diversity of plants with unique medicinal and pharmacological potential. The Ministry of AYUSH has provided the livelihood to tribal people by promoting Medicinal and Aromatic Plants (MAPs). Germany regulatory authority's herbal watchdog agency called "Commission E" carried out extensive literature surveys on 300 common medicinal herbs, assessing the eminence and uses of medicinally useful herbs. The enrichment of folk medicine was undertaken by reputed companies like the American Herbal Pharmacopoeia, American Botanical Council, and British Herbal Compendium. The Indian government has documented the Indian folklore knowledge, which can be obtained in the public domain and maintains the autonomy of this folklore knowledge and safeguards it from being misused in patenting on non-patentable inventions.

The "Traditional Knowledge Digital Library" (TKDL) is overseen by national and international IPR laws which is maintained and developed by the government with a joint collaboration with AYUSH and CSIR and is an exclusive digital repository of folklore medicine of India. The database is available online and is publically available and documents the in-depth information about the herbal formulations of Ayurveda, Siddha, Unani, and Yoga. The vital information in the database contains plants' names, therapeutic formulations, and Ayurvedic narration of diseases along with their contemporary names (Mukherjee et al. 2007). TKDL automatically converts the information from Sanskrit into various languages.

The utilization of recent technology like nanotechnology in the field of conventional, indigenous, or folkloric medicines has been endorsed by practitioners

worldwide. Due to the lacuna in scientific justification and difficulties in processing of folklore medicines, the novel formulation development was not being considered for a long time. By developing novel drug delivery systems (NDDS) like microspheres, nanoparticles, matrix system, liposomes, and solid-lipid nanoparticles, the technical necessities of folklore medicine can be gratified by modern phytopharmaceutical research. In most of the conventional dosage forms depending on physicochemical and biochemical properties, an inadequate amount of drug administered reaches the targeted site, a greater amount of the drugs gets distributed all over the body resulting in meager therapeutic effect (Sharma et al. 2011). NDDS have potential advantage including drug-targeted delivery, thereby reducing the dosage frequency with an increased solubility, absorption, and decreased elimination (Yadav et al. 2011). Among all the NDDS, nanoparticles are considered to be a potential drug delivery system.

Nanotechnology is the creation, manipulation, and use of materials at the nanometer size scale (1 to 100 nm). Norio Taniguchi in 1974 introduced the term "nanotechnology" for the first time at the International Conference on Industrial Production in Tokyo, in order to demonstrate the super thin processing of materials with nanometer precision and the construction of nanosized mechanisms. In a lecture by Richard Feynman (known as the "father of nanotechnology") in 1959, the ideas of nanotechnological approaches were promoted, at the session of the American Physical Society, and later on in 1986, his ideas were developed by Eric Drexler. In the early 1980s, with the invention of the scanning tunneling microscope (STM), nanotechnology and nanoscience got an enhancement and led to the innovation of Fullerenes in 1985 and the structural assignment of carbon nanotubes in 1991.

Nanotechnology has a greater impact in the field of molecular manipulations whereby powder forms of the huge materials are synthesized which acquires novel properties. Such products occupy a quantum of space (<1–100 nanometers) and have prospective and precision in terms of performance, hence accepted worldwide, and applications include various fields like pharmacy, engineering, agriculture, health and medicine, economy, and day-to-day life (Nikalje 2015).

Even though nanoparticles are considered as the discovery of modern science, they have been documented in various ancient scriptures for thousands of years (Brill and Cahill 1988). The advancement in technologies like the synthesis and application of nanoparticles are always considered to be beneficial for humans. The synthesis of bionanoparticles is mainly dependent on medicinal plants which have the capability to integrate with metal ions and reduce them to metallic nanoparticles. The application of nanoparticles in folklore medicine when compared to allopathic medicine offers numerous advantages such as drug-targeted delivery, minimization of dose, cost-effectiveness, nontoxic, and environmental-friendly. The utilization of medicinal plant extracts for the biosynthesis of nanoparticles is primarily under exploitation.

The current review attempts to highlight the potential use of nanoparticles as biotherapeutics in folklore medicine. The review describes the different types of nanoparticles/nano drug delivery systems, properties, their biosynthesis, therapeutic

activity, advantages, and disadvantages. Hence, this concept can be defined as traditional herbal medicine manipulated at the nanoscale level and integrated with folklore herbs for their potential therapeutic value for prospective drug development to combat diseases and for the proper physiology of the body.

4.2 Medicinal Plants Used in Folklore Medicine and Its Importance

In India, the most primitive records of the use of medicinal plants, documented in the Rigveda, elaborate the therapeutic properties of some herbs. Numerous medicinal plants are found to be valuable in the management and of diseases, and the medicinal properties of these folklore medications have been documented in ancient Indian literature. Beneficial medicinal activities of numerous plant species, which are rich in secondary metabolites, have been reported from various plant species which can be used to manage health. The folkloric or tribal system and Indian medicine system akin to Ayurveda, Siddha, and Unani comprehensively use medicinal plants for the well-being management all over the world. It can be predicted that nearly 75% of plant-based therapeutic entities used worldwide were included from traditional/folk medicine.

In India, nearly 70% of the modern drugs are prepared from natural resources, and a number of other synthetic analogs have been prepared from prototype compounds isolated from plants. It was reported that more than 60% of cancer drug available in the market are plant derivative (Sen et al. 2009; Pan et al. 2014). The WHO has estimated the demand for medicinal herbs and their derivatives have a profound value in the market which is around $14 billion per annum, and the demand is steeply increasing at the rate of 15–25% annually. By 2050, the trade of medicinal plants will grow up to US$ 5 trillion as estimated by the WHO.

Folklore healers from various tribes utilize up to 2500 plant species in their treatment and almost 100 species of plants which serve as a regular source of medicines in India. Sankaranarayanan (1988) documented the folklore medicines for jaundice from Coimbatore and Palghat districts of Tamilnadu and Kerala, India. Devendra et al. (2010) conducted a survey on folklore medicinal plants of Gulbarga district, Karnataka, India, and information on 36 plant species from 34 genera and 23 families. The therapeutic implications of these plants include anticancer, antidiabetic, analgesic, hepato- and cardioprotective, antispasmodic, reproductive health, and a wide range of other pharmacological properties (D'Cruz et al. 2010; Lohiya et al. 2016). Many folkloric drugs are food products which are known to have a beneficial effect on male reproductive health, including Gokhru or land caltrops (*Tribulus terrestris*), Tongkat Ali (*Eurycoma longifolia*), garlic (*Allium sativum*), onion (*Allium cepa*), marijuana (*Cannabis sativa*), chili pepper (*Capsicum frutescens*), and ginger (*Zingiber officinale*) (Tambi et al. 2012; Henkel et al. 2014; Lohiya et al. 2016; Mansouri et al. 2016). Renowned herb *Withania somnifera*, popularly known as Ashwagandha or the "Indian ginseng" is regularly used in the treatment of male infertility (Lohiya et al. 2016; Malviya et al. 2016).

The utilization of herbal medicine has been resurfacing owing to the side effects of contemporary drugs, failure of contemporary treatment against chronic diseases, and microbial resistance (Sen et al. 2010; Pan et al. 2014). The use of these herbal remedies is not only cost-effective but also safe and almost free from deleterious effects. The village elders, farmers, and tribal from several countries including China, Middle East, Africa, Egypt, South America, and other developing countries of the world have a good knowledge about well-being which started thousands of years ago and is still part of medical practices by folks of various regions of Indian subcontinents as well. Ravishankar and Shukla (2007) enlisted the medicinal plants of India and their therapeutic uses (Table 4.1).

4.3 Nanotechnology in Herbal Medicines

The potential impact of novel nano-herbal formulations in therapeutics and disease prevention is foreseen to change healthcare in an essential way. Additionally, therapeutics can be customized to every patient's profile.

The synergistic activity of herbal medicines relies on phytochemicals, rich in secondary metabolites, which improves its therapeutic value. However, most of the herbal medicines have poor solubility leading to deprived bioavailability and increased systemic clearance requiring frequent application or higher dose, making the drug a poor contender for therapeutic use. Nanotechnology and herbal science are integrated to overcome the limitations of using herbal drugs in a scientific way, for the development of novel drug delivery system which includes nano dose which aids in increasing the biosolubility and bioavailability, protection from toxicity, and persistent delivery. The novel drug delivery systems have predestined rates and site precise action.

In novel drug technology, the flow of drug circulation is aided by blending the drug into nanocarriers. Secure materials, including biodegradable polymers, lipids, and polysaccharides, are used in the preparation of nanocarriers. Nanocarriers provide the best possible surface area and maximize solubility, bioavailability, and help in accurate drug targeting when compared to micrometer-sized carriers. The amount of drug included into nanocarriers is lesser when compared to encapsulated form. Consequently, for enhancing the activity and to overcome the problems related with plant medicines, the nanosized drug delivery systems (NDDS) of herbal drugs have a potential future. The various herbal nanoparticles delivery systems are summarized in Table 4.2.

Among the various novel drug delivery systems, nanoparticles can be designed to target an individual organ which improves the selectivity, drug delivery, effectiveness, and safety, thus reducing the dose requirement and increasing patient compliance. The requirement of an ideal nanoparticulate system is that it should circulate in the bloodstream and should be small enough to reach the target cells and tissues. Nanoparticles are present in large amounts in the human body even at the cellular level; hence, it is assumed that nanoparticles can be employed to treat severe,

Table 4.1 List of some well-known Indian medicinal plants and their uses (Ravishankar and Shukla 2007)

Species name	Family	Parts used	Therapeutic uses	References
Acorus calamus	Araceae	Rhizome	Nervine tonic, antispasmodic	Bose et al. (1960); Satyavati et al. (1976)
Aegle marmelos	Rutaceae	Fruit	Hypoglycemic, chemopreventive	Vyas et al. (1979); Dixit et al. (2006)
Allium sativum	Alliaceae	Bulbs	Anti-inflammatory, antihyperlipidemic, fibrinolytic	Dixit et al. (2006)
Aloe barbadensis	Alliaceae	Gel	Skin diseases—mild sunburn, frostbite, scalds; wound healing	Baliga (2006)
Andrographis paniculata	Acanthaceae	Whole plant	Cold, flu, hepatoprotection	Koul and Kapil (1994)
Asparagus racemosus	Alliaceae	Roots	Adaptogen, galactogogue	Dahanukar et al. (1997); Gupta and Mishra (2006)
Bacopa monnieri	Scrophulariaceae	Whole plant	Antioxidant, memory enhancing	Singh and Dhawan (1997)
Berberis aristata	Berberidaceae	Bark, fruit, root, stem, wood	Antiprotozoal, hypoglycemic, anti-trachoma	Dutta and Iyer (1968)
Boerhavia diffusa	Nyctaginaceae	Roots	Diuretic, anti-inflammatory and anti-arthritic	Harvey (1966)
Boswellia serrata	Burseraceae	Oleo resin	Anti-rheumatic, anti-colitis, anti-inflammatory and anticancer	Sharma et al. (2000c)
Butea monosperma	Fabaceae	Bark, leaves, flowers, seeds and gum	Adaptogen, abortifacient, antiestrogenic, anti-gout, anti-ovulatory	Sharma et al. (2000d)
Calotropis gigantea	Asclepiadaceae	Flowers, whole plant, root, leaf	Anti-inflammatory, spasmolytic, asthma	Sharma et al. (2000e)
Callicarpa macrophylla	Verbenaceae	Leaves, roots	Uterine disorders	Sood (1995)
Cassia fistula Linn.		Resin	Laxative, antipyretic, worm infestation	Joshi (1998)
Centella asiatica	Umbelliferae	Whole plant	Tranquilizer, memory enhancer, wound healing	Suguna et al. (1996)
Chlorophytum borivilianum	Alliaceae	Roots	Aphrodisiac	Farooqi et al. (2001)

(continued)

Table 4.1 (continued)

Species name	Family	Parts used	Therapeutic uses	References
Cissus quadrangularis	Vitaceae	Whole plant, root, stem and leaf	Bone fracture, inflammation	Udupa and Prasad (1964); Deka et al. (1994)
Clerodendrum serratum	Verbenaceae	Root, leaf, Stem	Malaria, anti-asthmatic, anti-allergic	Gupta and Gupta (1967)
Commiphora mukul	Burseraceae	Resin	Hypolipidemic, obesity, rheumatoid arthritis	Satyavati (1991)
Crataeva nurvala	Capparidaceae	Stem bark, leaf	Urinary disorders including stones	Anand et al. (1995)
Crocus sativus	Iridaceae	Stigma	Aphrodisiac, anti-stress, antioxidant	Billore et al. (2004a)
Curculigo orchioides	Amaryllidaceae	Root stock	Spermatogenesis enhancer	Joshi (2005)
Curcuma longa	Zingiberaceae	Rhizome	Anti-inflammatory, wound healing enhancer, chemopreventive agent, antioxidant, anticancer	Tripathi et al. (1973); Narasimhan et al. (2006)
Desmodium gangeticum	Papilionaceae	Root	Antioxidant, anti-rheumatic	Govindarajan and Vijayakumar (2006)
Eclipta alba	Compositae	Whole plant	Hepatoprotecive/promotes hair growth	Chandra (1978)
Eugenia jambolana	Myrtaceae	Seed, bark, leaf	Hypoglycemic, anti-inflammatory, antidiarrheal, antipyretic	Sharma et al. (2001)
Ficus religiosa	Urticaceae	Bark	Anti-ulcer (gastric ulcer); anti-inflammatory, hypoglycemic agent	Ambike and Rao (1967)
Gymnema sylvestre	Asclepiadaceae	Roots and leaves	Antidiabetic, antihyperglycemic	Narasimhan et al. (2006)
Gloriosa superba	Liliaceae	Tuber	Spasmolytic, oxytocic, source plant for colchicine	Sharma et al. (2002a)
Glycyrrhiza glabra	Papilionaceae	Stem	Expectorant, peptic ulcer treatment	Mitra and Rangesh (2004a)
Hedychium spicatum	Zingiberaceae	Rhizome	Soothening, expectorant, antitussive, anti-asthmatic	Chaturvedi and Sharma (1975)
Hippophae rhamnoides	Elaeagnaceae	Fruits	Extensively used in the treatment of circulatory disorders, wound healing enhancer, duodenal ulcer	Arora et al. (2006)
Holarrhena antidysenterica	Apocynaceae	Stem bark, leaf, seed	Antispasmodic, anti-colitis, hypoglycemic	Mitra and Rangesh (2004b)
Inula racemosa	Asteraceae; Compositae	Roots	Used in gastro intestinal disorders, diuretic, expectorant and allergic disorders	Mishra (2004a)

Leptadenia reticulata	Asclepiadaceae	Root, leaf, fruit	Galactogogue, vasodilator, anabolic	Anjaria et al. (1975)
Momordica charantia	Cucurbitaceae	Root, leaf, fruit, seed	Antidiabetic	Ahmed et al. (2001)
Oroxylum indicum	Bignoniaceae	Root, root bark, leaf, fruit, seed	Anti-inflammatory, diuretic	Gujral et al. (1955)
Phyllanthus amarus	Euphorbiaceae	Whole plant	Hepatoprotective	Balachandran and Govindarajan (2004)
Picrorhiza kurroa	Scrophulariaceae	Tubers	Hepatoprotective, adaptogen	Narasimhan et al. (2006)
Plumbago zeylanica	Plumbaginaceae	Root, root bark	Antipyretic, anticancer, anticoagulant, cytotoxic	Krishnaswamy and Purushothaman (1980)
Pueraria tuberosa	Fabaceae	Tuberous root	Anti-implantation, estrogenic, anti-inflammatory, dysmenorrhea, DUB	Billore et al. (2004b)
Rubia cordifolia	Rubiaceae	Root	Anti-inflammatory, antitumor, hypoglycemic	Sharma et al. (2002c)
Saussurea lappa	Asteraceae	Roots	Analgesic, aphrodisiac, asthma	Chaurasia (2006)
Swertia chirata	Gentianaceae	Whole plant	Antimalarial, hypoglycemic, febrifuge	Hamsaveni et al. (1981); Dixit et al. (2006)
Symplocos racemosa	Symplocaceae	Bark	Antidiarrheal	Sharma et al. (2002d)
Taxus baccata	Taxaceae	Source of taxol	Used in the treatment of metastatic breast cancer	Chauhan et al. (2006)
Tecomella undulata	Bignoniaceae	Bark, seeds	Antibacterial, hypoglycemic, hepatoprotective	Billore et al. (2004c)
Terminalia chebula	Combretaceae	Fruits	Laxative, antioxidant	Narasimhan et al. (2006)
Tinospora cordifolia	Menispermaceae	Stem	Adaptogen, immunomodulator	Thatte et al. (1994); Dahanukar et al. (1997)
Tribulus terrestris	Zygophyllaceae	Whole plant	Diuretic, anti-urolithiatic, cytoprotective	Chakraborty and Neogi (1978); Sangeeta et al. (1993)
Vitex negundo	Verbenaceae	Leaves, root, bark, flowers, seed	Anti-inflammatory, anti-arthritic, immunomodulator	Nair and Saraf (1995)
Withania somnifera	Solanaceae	Root	Adaptogen, antirheumatic	Sandhya and Sushil (1998)
Zingiber officinale	Zingiberaceae	Rhizome	Fever, cough, asthma, antiemetic	Sharma et al. (2002b)

Table 4.2 Herbal drug nanoparticles (Ansari et al. 2012)

Formulations	Active ingredients	Biological activity	Method of preparation	References
Artemisinin nanocapsules	Artemisinin	Anticancer	Self-assembly procedure	Chen et al. (2009)
Berberine-loaded nanoparticles	Berberine	Anticancer	Ionic gelation method	Chang et al. (2011)
Curcuminoids solid lipid nanoparticles	Curcuminoids	Anticancer and antioxidant	Micro-emulsion technique	Nayak et al. (2010)
Glycyrrhizic acid loaded nanoparticles	Glycyrrhizin acid	Anti-inflammatory antihypertensive	Rotary-evaporated film ultrasonication method	Hou and Zhou (2008)
Nanoparticles of *Cuscuta chinensis*	Flavonoids and lignans	Hepatoprotective and antioxidant effects	Nanosuspension method	Yen et al. (2008)

chronic diseases and genetic disorders. Herbal medicines can be targeted to various organs such as the brain, lungs, liver, kidneys, and gastrointestinal tract (Kostarelos 2003; Allen and Cullis 2004).

4.4 Nanoparticles in Folklore Medicines

Nanoparticles are characterized as powdered materials with particles measuring less than 100 nanometers. This diminishment in scale brings about radical changes in their physical properties compared to bulky objects. They can be metallic, mineral, polymer-based, or a blend of materials and are used in various fields like medicine, nutrition, and energy (Chandran et al. 2006). Herbal drug solubility and efficacy can be improved by utilizing nanoparticles, thereby localizing the drug at a specific site. The rich biodiversity and accessibility of plant substances have led to the increasing use of plants for the synthesis of nanomaterial blend (Mondal et al. 2011).

Nanoparticles of diverse size and shape have been biosynthesized from a range of parts of plants like seeds, leaves, fruits, flowers, stems, and roots (Prasad 2014, Prasad et al. 2018). Nanomedicine has a massive potential in curing different interminable maladie and a huge role to play in the healthcare sector in treating various chronic diseases (Cruz et al. 2010). Secondary active metabolites of plants, viz., flavonoids, tannins, and terpenoids, are highly soluble in water. Because of their high molecular weight, the absorption rates are low; hence, they are unable to cross the lipid membranes of the cells resulting in the loss of bioavailability and effectiveness. Some herbal extracts are not used clinically due to these practical problems. Encapsulating these herbal secondary metabolites in folklore medicine with nanoparticles/nanomaterials will solve these practical problems.

For effectual therapeutic activity of folklore medicine, the nanoparticles must have a size range of <50 nm, helping them to penetrate the body rapidly, and the general routes of entry are through the oral and mucous membranes which will activate diverse reactions. These nanoparticles often concentrate in sites other than those therapeutically anticipated and are vehicles for drug delivery into the body. All through the entire treatment phase, nanosystems can convey these active constituents at an optimum concentration, directing it to the preferred action site. Conventional folklore treatments do not meet these requirements.

4.5 Different Classes of Nanoparticles

Nanomaterials can be broadly classified into inorganic and organic. Organic polymers (organic nanoparticles) and/or inorganic elements (inorganic nanoparticles) are widely used to prepare nanoparticles. Liposomes, dendrimers, carbon nanomaterials, and polymeric micelles are examples of organic nanoparticles (Fig. 4.1).

4.5.1 Inorganic Nanoparticles

Inorganic nanoparticles have a central core made of inorganic materials that describe their fluorescent, magnetic, electronic, and optical properties. The different types of inorganic particles include magnetic, metallic, ceramic, and nanoshells; their description and size are depicted in Table 4.3.

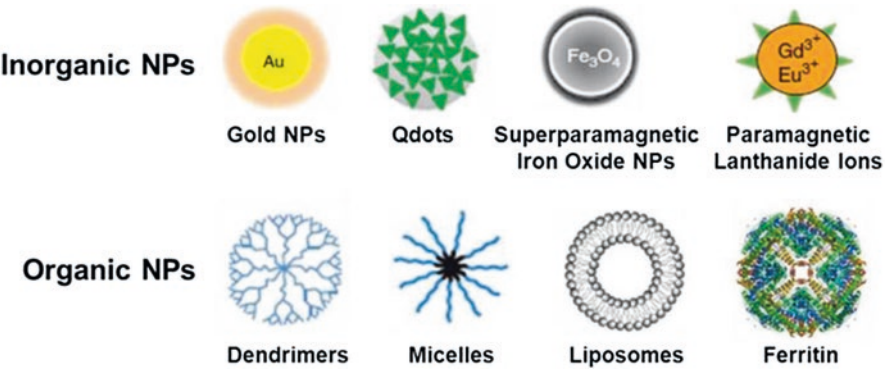

Fig. 4.1 Different types of organic and inorganic nanoparticles (https://www.nanoshel.com/organic-and-inorganic-nanoparticles)

Table 4.3 Inorganic nanoparticles

Inorganic nanomaterial	Description	Size (nm)
Ceramics	Nanoscale ceramics such as hydroxyapatite (HA), zirconia (ZrO_2), silica (SiO_2), titanium oxide (TiO_2), and alumina (Al_2O_3)	<100
Magnetic	Superparamagnetic iron oxide particles	5–100
Metallic	Gold, silver, zinc, copper particles	1–100
Nanoshells	Spherical core of a compound surrounded by a shell or outer coating of thin layer of another material	1–20

Table 4.4 Organic nanoparticles

Organic nanomaterial	Description	Size (nm)
Carbon tubes	Coaxial graphite sheets rolled up into cylinders	<100 nm
Dendrimers	Dendrimers are highly branched synthetic polymers with layered architectures constituted of a central core	<15
Liposomes	Liposomes are phospholipid vesicles that have a bilayer membrane structure	50–100
Polymers	Polymers are macromolecules composed of a large number of repeating units organized in a chain-like molecular architecture	10–1000
Quantum dots	Colloidal fluorescent semiconductor nanocrystals	2–10

4.5.2 Organic Nanoparticles

The various types of organic nanoparticles, namely, carbon nanotubes, quantum dots, dendrimers, liposome, and polymers, and their description and size are described in Table 4.4.

4.5.3 Ceramic Nanoparticles

Currently, the utilization of new ceramic materials for biomedical research has grown steadily, due to improvisation of the physicochemical properties and reduction in the cytotoxicity of biological systems; synthetic nanoscale ceramics such as alumina (Al_2O_3), hydroxyapatite (HA), silica (SiO_2), titanium oxide (TiO_2), and zirconia (ZrO_2) are being synthesized.

The employment of ceramic nanoparticles for the controlled release of drugs is one of the most exploited areas in folklore medicine. In this field, the dose and size are important. Nanoparticles in drug delivery have high stability, high load capacity,

easy incorporation into hydrophobic and hydrophilic systems, and different routes of administration (oral, inhalation). In addition, a variety of organic groups which may be functionalized on its surfaces allow for a directed effect (Fadeel and Garcia-Bennett 2010).

4.5.4 Magnetic-Based

Magnetic nanoparticles can be manipulated using magnetic fields; they belong to a class of a nanoscale particle consisting of magnetic elements like iron, nickel, and cobalt, plus their chemical compounds. Recently, magnetic nanoparticles have been used in scientific research on folklore medicine as they possess unique properties that have use in medicine. Magnetic nanoparticle clusters are composed of a number of individual magnetic nanoparticles are known as magnetic nanobeads, with a diameter of 50–200 nm (Tadic et al. 2014). Magnetic nanoparticle clusters are a basis for their further magnetic assembly into magnetic nanochains (Kralj and Makovec 2015).

4.5.5 Metal-Based Materials

Metal nanoparticles are nanosized metals with a size range of 1–100 nm. Faraday in 1857 first recognized the existence of metallic nanoparticles in solution. For improving the efficacy of folklore medication, the utilization of noble metals like gold, silver, zinc, copper, and metal oxides, such as titanium dioxide, can be used (Table 4.5).

4.5.6 Nanoshells

Nanoshells consist of a spherical core of a complex bounded by a shell or outer coating of thin layer with an additional material of 1–20 nm thick which can be synthesized from semiconductors, metals, or insulators. Core-to-shell ratio used by the materials determines the property of nanoshells. Dielectric core enclosed by metallic shell, especially gold (AuNSs). In these cases, drug is encapsulated or adsorbed onto the shell surface via specific functional groups or by electrostatic stabilization. AuNSs are employed to deliver antitumor drugs (e.g., doxorubicin, paclitaxel, small interfering RNA, and single-stranded DNA) into cancer cells, which augment the efficacy of treatment. AuNSs can also be functionalized with active targeting ligands, such as antibodies, aptamers, and peptides to increase the particle's specific binding to the desired targets (Mudshinge et al. 2011).

Table 4.5 Summary of plant-derived metallic nanoparticles and its biomedical applications (Kuppuswamy et al. 2016)

Plants used	Nanoparticles	Parts of plant	Size (nm)	Shapes	Plant metabolites involved in bioreduction	Pharmacological applications	Reference
Acalypha indica	Ag, Au	Leaves	20–30	Spherical	Quercetin, plant pigment	Antibacterial	Krishnaraj et al. (2010)
Aloe vera	In_2O_3	Leaf	5–50	Spherical	Biomolecules	Optical properties	Maensiri et al. (2008)
Alternanthera sessilis	Ag	Whole	40	Spherical	Amine, carboxyl group	Antioxidant, antimicrobial	Niraimathi et al. (2013)
Andrographis paniculata	Ag	Leaves	67–88	Spherical	Alkaloids, flavonoids	Hepatocurative activity	Suriyakalaa et al. (2013)
Artemisia nilagirica	Ag	Leaves	70–90	Spherical	Secondary metabolites	Antimicrobial	Song and Kim (2008)
Boswellia serrata	Ag	Gum	7–10	Spherical	Protein, enzyme	Antibacterial	Kora et al. (2012)
Caria papaya	Ag	Fruit	15	Spherical	Hydroxyl flavones, catechins	Antimicrobial	Jain et al. (2009)
Cassia fistula	Au	Stem	55–98	Spherical	Hydroxyl group	Antihypoglycemic	Daisy and Saipriya (2012)
Cinnamon zeylanicum	Ag	Leaves	45	Spherical	Water-soluble organics	Antibacterial	Sathishkumar et al. (2009)
Citrullus colocynthis	Ag	Calli	5–70	Triangle	Polyphenols	Antioxidant, anticancer	Satyavani et al. (2011)
Citrus sinensis	Ag	Peel	35	Spherical	Water-soluble compounds	Antibacterial	Kaviya et al. (2011)
Dillenia indica	Ag	Fruit	11–24	Spherical	Biomolecules	Antibacterial	Singh et al. (2013)
Dioscorea bulbifera	Ag	Tuber	8–20	Rod, triangular	Diosgenin, ascorbic acid	Antimicrobial	Ghosh et al. (2012)
Euphorbia prostrata	Ag	Leaves	52	Rod, spherical	Protein, polyphenols	Antiplasmodial	Zahir and Rahuman (2012)
Gelsemium sempervirens	Ag	whole	112	Spherical	Protein, amide, amine group	Cytotoxicity	Das et al. (2011)
Lippia citriodora	Ag	Leaves	15–30	Spherical,	Isoverbascoside compound	Antimicrobial	Cruz et al. (2010)

Mentha piperita	Au, Ag	Leaves	90–150	Spherical	Menthol	Antibacterial	MubarakAli et al. (2011)
Mirabilis jalapa	Au	Flowers	~100	Spherical	Polysaccharides	Antimicrobial	Vankar and Bajpai (2010)
H. canadensis	Ag	Whole	113	Spherical	Phenolics, protein	Cytotoxicity	Das et al. (2011)
Iresine herbstii	Ag	Leaves	44–64	Cubic	Biomolecules phenolic compound	Biological activities	Dipankar and Murugan (2012)
Melia azedarach	Ag	Leaves	78	Irregular	Tannic acid, polyphenols	Cytotoxicity	Sukirtha et al. (2012)
Tinospora cordifolia	Ag	Leaves	34	Spherical	Phenolic compound	Antilarvicidal	Jayaseelan et al. (2011)
Trigonella foenum-graecum	Au	Seed	15–25	Spherical	Flavonoids	Catalytic	Aromal and Philip (2012)
Withania somnifera	Ag	Leaves	5–40	Irregular, spherical	Methyl-7-oxooctadecanoate	Antimicrobial	Nagati et al. (2012)

4.5.7 Carbon-Based Materials

Carbon nanotubes are made up of coaxial graphite sheets (<100 nm) which are rolled up into cylinders. They belong to the family of fullerenes, formed either as single- (one graphite sheet) or multi-walled nanotubes (numerous concentric graphite sheets). They demonstrate exceptional strength and electrical properties and are very good thermal conductors. Due to their metallic or semiconductor nature, nanotubes are frequently used as biosensors. Surface functionalization renders the carbon nanotubes water soluble. Hence, they are used as drug carriers and tissue-repair scaffolds. These nanomaterials are mainly made up of carbon made into the form of a hollow spheres, ellipsoids, or tubes. Spherical and ellipsoidal carbon nanomaterials are referred to as fullerenes, while cylindrical ones are called nanotubes.

4.5.8 Dendrimers

Dendrimers have a central core which are highly branched synthetic polymers (<15 nm) with layered architectures. The surface of a dendrimer has numerous chain ends, which can be tailored to perform specific chemical functions. This property could also be useful for catalysis as three-dimensional dendrimers contain interior cavities into which other molecules could be placed, they may be useful for drug delivery. Dendrimers demonstrate intrinsic drug properties and are used as tissue-repair scaffolds. Furthermore, dendrimers are excellent drug and diagnostic imaging agent.

4.5.9 Liposome-Based

Liposomes are phospholipid vesicles (50–100 nm) made up of a bilayer membrane structure comparable to that of biological membranes having an internal aqueous phase. Based on the size and number of layers, liposomes are classified into multi-oligo or uni-lamellar. Liposomes are nanoparticulate systems that have been developed for more than four decades for drug delivery to a specific site in the body (Samaligy et al. 2006).

Their amphiphilic nature allows them to convey hydrophilic drugs entangled within their aqueous interior and hydrophobic drugs dissolved into the membrane. Liposomes demonstrate exceptional penetration, circulation, and diffusion properties, and the liposome surface can also be customized with ligands and/or polymers to boost the specificity of drug delivery.

4.5.10 Polymeric Nanoparticles

Polymeric nanoparticles are macromolecules made up of repeated subunits of chain-like molecular architecture which brings about the variations in composition, structure, and properties. A polymeric nanoparticle ranges from 10–1000 nm in diameter, protecting drugs efficiently. They can materialize as nanocapsules (NCs) and nanospheres (NSs); these structures have varied composition and structural organization. Nanocapsules enclose an oily core bounded by a polymeric membrane; the active ingredient can be adsorbed to the polymeric membrane and/or dissolved in the oily core. This active component has better adsorption on nanospheres manufactured from polymeric substrates. Although there is an increasing demand for the new types of polymers, some of them have already been used extensively for polymeric nanoparticles, including poly-L-lactic acid (PLA) and copolymers with glycolic acid (PLGA) (Schaffazick et al. 2003; Alexis et al. 2008; Ajazuddin 2010; Prasad et al. 2017). Because of this diversity of structures, properties, and compositions, polymers are mainly utilized in nanoparticle systems to produce nanoparticles suitable for a specific folklore medication.

4.5.11 Natural and Synthetic Polymeric Nanoparticles

Chitosan, albumin, and heparin are the naturally occurring nanoparticles which have been extensively used for the delivery of drugs, DNA, and proteins. In addition, the use of hydroxypropyl-methacrylamide (HPMA), polyethylene glycol (PEG), polylactic acid-glycolic acid (PLGA), and polylactic acid (PLA) nanoparticles as natural and synthetic polymers has been recognized. The use of conjugated polymeric nanoparticles with chemotherapeutic drugs can minimize the damaging effects of the free drug administration (Cho et al. 2008).

4.5.12 Quantum Dots

Quantum dots are photostable colloidal fluorescent semiconductor nanocrystals (2–10 nm). The central core of quantum dots is made up of an amalgamation of elements from groups II–VI of the periodic system (CdSe, CdTe, CdS, PbSe, ZnS, and ZnSe) or III–V (GaAs, GaN, InP, and InAs), which are coated with a layer of ZnS. They demonstrate high quantum yield and composition-tunable emission spectra and are extremely resistant to photo and chemical degradation. All these features make quantum dots good candidates for drug delivery in folklore medicine.

4.6 Choice of Nanoparticles in Folklore Medicine

The application of metallic nanoparticles in folklore medicine has gained a tremendous interest due to the emerging significance of targeted drug delivery of medicine. Primitive Indian medical sciences made use of herbs or an amalgamation of mineral-herb preparations. Gold, silver, lead, tin, copper, iron, mercury, and zinc are the most commonly used therapeutic metals used in herbal medicines. The term "Bhasmikaran" often used in Indian medicine refers to the transition of non-biocompatible herbo-mineral material to a biocompatible form. The process of preparation of Bhasma (metal ash) enhances the medicinal value by transforming the metal from to its zero-valent state to an advanced oxidation state forming a metal oxide. These metals are maximally processed and are changed into their therapeutic form which can be utilized to enhance the medicinal value of folklore medicine.

In conservative Ayurvedic practices and medicine, "Swarna Bhasma" aka "gold ash" is frequently given as a potent treatment. Gold ash has been utilized in various folklore medicines to improve human health. Nanosized gold particles, oxidized form of gold ash, has superior medicinal properties when compared to the native state metallic gold. In Indian literature of 2500 BC, there occurs evidence of the therapeutic use of gold nanoparticles, and these practices are still being followed to date. There are great prospects for gold nanoparticles (GNPs) to be used in human health and cosmetics applications (Alanazi et al. 2010).

Gold nanoparticles have been extensively employed in biomedical applications (Bhattacharya and Mukherjee 2008; Sperling et al. 2008; Puvanakrishnan et al. 2012), separation sciences (Sýkora et al. 2010), disease diagnostics (Torres-Chavolla et al. 2010), and pharmaceuticals (Bhumkar et al. 2007; Cai et al. 2008).

The antibacterial and anti-inflammatory properties of silver nanoparticles promote faster wound healing. Because of these attributes, silver nanoparticles have been integrated into the already available wound dressings, antiseptic formulations, and medical implant coatings (Cohen et al. 2007; Huang et al. 2007; Asha Rani et al. 2008; Cox et al. 2011; Li et al. 2011a, b; Pollini et al. 2011; Aziz et al. 2016).

Platinum nanoparticles have been employed extensively in biomedical applications in either pure or alloyed form with other nanoparticles (Hrapovic et al. 2004) and palladium nanoparticles in catalysis and electro-catalysis applications (Akhtar et al. 2013; Gopidas et al. 2003), chemical sensors (Coccia et al. 2012), optoelectronics (Chen et al. 2007), and antibacterial applications (West et al. 2010).

Metallic nanoparticles including iron (Pankhurst et al. 2003; Njagi et al. 2010), copper (Lee et al. 2011; Yadav et al. 2017), zinc oxide (Brayner et al. 2006), and selenium (Prasad et al. 2013) have applications in medical treatments and antimicrobial formulations.

Numerous diseases from infertility to asthma, diabetes, and cancer can be treated by administering gold nanoparticles along with herbs. The gold acting as a catalyst transports the herbal medicine to the target organ thereby enhancing its activity and also helping to restore the normal function of the diseased organ.

Phenolic acids, flavonoids, alkaloids, and terpenoids are secondary metabolites found in crude plant extracts, and these biocompounds are primarily used in the ionic reduction of bulk metallic nanoparticles (Aromal and Philip 2012). Eco-friendly nanoparticles produced from these primary and secondary metabolites are involved in the redox reaction. Numerous reports showed that the biosynthesized nanoparticles effectively controlled oxidative stress, genotoxicity, and apoptosis-associated changes (Kim et al. 2007). In the field of agriculture industry and plant sciences, nanoparticles are being used to convert the agricultural and food wastes into energy and useful by-products.

4.7 Need of the Nanoparticles in Folklore Remedies

To overcome the flaws of using traditional herbal drugs, nanoparticles are incorporated due to the following reasons: Nanoparticles can target the herbal medicine to specific organ, improving the selectivity, drug delivery, effectiveness, and safety. Nanoparticles improve the efficacy by increasing the solubility of herbal drug and target the drug to a specific site (Sharma et al. 2014). Owing to their unique size and high-loading capacities, nanoparticles can convey high concentrations of drugs to disease sites. Delivering the drug in nanosize boosts the surface area of the drugs consequently, allowing rapid distribution in the bloodstream, and shows enhanced permeation and retention effect, i.e., improved permeation through the barriers and retention due to meager lymphatic drainage (Jayaseelan et al. 2013). Displays decreased the side effects by submissively targeting the site of action without the addition of any particular ligand moiety (Ansari et al. 2012).

4.8 Mechanism of Nanoparticle Formation

A number of methods are used for the synthesis of nanoparticles (NPs) including physical, chemical, enzymatic, and biological. Physical methods include plasma arcing, ball milling, thermal evaporate, spray pyrolysis, ultrathin films, pulsed laser desorption, lithographic techniques, sputter deposition, layer-by-layer growth, molecular beam epistaxis, and diffusion flame synthesis of nanoparticles (Joerger et al. 2000), while chemical methods used are electrodeposition, sol-gel process, chemical solution deposition, chemical vapor deposition (Panigrahi et al. 2006; Oliveira et al. 2005), soft chemical method, Langmuir Blodgett method, catalytic route, hydrolysis (Pileni 1997), co-precipitation method, and wet chemical method (Gan et al. 2012). Physical and chemical methods are hazardous to the environment and to human health because of the use of high radiation and highly concentrated reductants and stabilizing agents. A single-step bioreduction process is involved in the biosynthesis of nanoparticles; hence, a lesser amount of energy is utilized to

synthesize eco-friendly NPs (Sathishkumar et al. 2009). Environmentally sustainable resources like plant extracts, bacteria, fungi, microalgae such as cyanobacteria, diatoms, seaweed (macroalgae), and enzymes (Iravani 2011) are used in biological methods (Prasad et al. 2016).

4.8.1 Bioreduction Mechanism

In the synthesis of nanoparticles, the intracellular or extracellular extract of organisms is merely combined with a solution of the metal salt at room temperature. The reaction is completed within minutes. The nature of the extract, its concentration, the concentration of the metal salt, the pH, temperature, and contact time all determine the rate of production of the nanoparticles, their quantity, and other characteristics. Figure 4.2 depicts the biosynthesis of nanoparticles using various organisms.

4.8.2 Plant-Mediated Synthesis of Nanoparticles

A plethora of organisms including fungi, bacteria, and yeast have been recognized for the synthesis of safe noble nanoparticles. But this is not industrially feasible as the microbial mediated synthesis of nanoparticles requires costly medium and aseptic conditions. Thus, the potential usage of plant systems for nanoparticle synthesis has garnered great interest (Prasad 2014). Henceforth, plant systems have

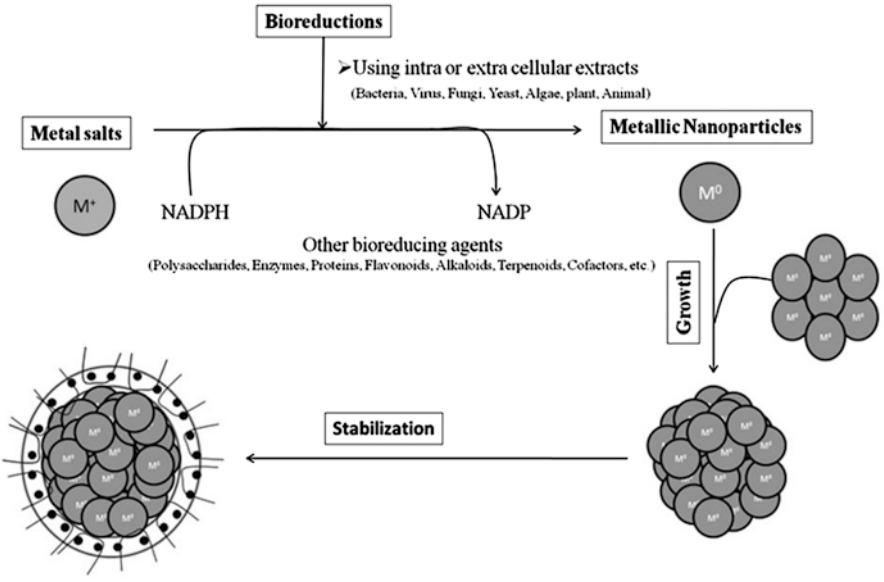

Fig. 4.2 The proposed mechanism of biological synthesis of nanoparticles (Velusamy et al. 2015)

been considered as ideal bioreactors for the synthesis of metal nanoparticles without using toxic chemicals.

Proposed mechanism of nanoparticle synthesis using plant extracts:

Gold: a number of biomolecules like proteins, sugars, amino acids, enzymes, and other traces of metals can be extracted from plants. These metabolites are involved in the bioreduction process. Thakkar et al. (2010) proposed the reaction of Au^{+} ions reduction into metallic Au nanoparticles in the presence of metabolites and redox enzymes. The reaction is given below:

$$\mathrm{HAu} + \mathrm{Cl_4 \cdot 4H_2O} + \text{plant extracts} \rightarrow \mathrm{AuoNPs} + \text{by} - \text{products}.$$

Platinum: platinum nanoparticles are produced by the general reaction below:

$$\mathrm{H_2Pt} + \mathrm{Cl_2 \cdot 6H_2O} + \text{plant extracts} \rightarrow \mathrm{PtoNPs} + \text{by} - \text{products}.$$

Copper: copper nanoparticles are synthesized from plant extracts, and the reduction mechanism was proposed by Ramanathan et al. (2013):

$$\mathrm{CuSO_4 \cdot 5H_2O} + \text{plant metabolites} \rightarrow \mathrm{CuoNPs} + \text{by} - \text{products}.$$

Silver: the biochemical reaction of $AgNO_3$ reacting with plant broth to produce silver nanoparticles was proposed by Tripathy et al. (2010):

$$\mathrm{Ag} + \mathrm{NO_3^-} + \text{plant extract} \rightarrow \mathrm{AgoNPs} + \text{by} - \text{products}.$$

4.8.3 Biological Synthesis of Metal Nanoparticles via Plants

Plants have long been known to have the potential to hyper-accumulate and reduce metallic ions (Kale et al. 2013). Plants are considered a more environment-friendly route for biologically synthesizing metallic nanoparticles and as detoxifying agents (Kale et al. 2013; Khan et al. 2013; Prasad 2014; Prasad et al. 2018). Bioactive alkaloids, phenolic acids, polyphenols, proteins, sugars, and terpenoids from plants have a pivotal role in first reducing the metallic ions and then stabilizing them (Marshall et al. 2007; Castro et al. 2011). The variability in the composition and concentration of these active biomolecules among different plants and their subsequent interaction with aqueous metal ions is said to contribute to the diversity of nanoparticle sizes and shapes produced. The synthesis of nanoparticles via plants by reducing metal salts is a direct process occurring at room temperature and starts by combining a plant extract sample with a solution containing metal salt. Biochemical reduction of the salts proceeds instantaneously, and the formation of nanoparticles is indicated by a change in the color of reaction mixture. An initial activation period occurs during the synthesis, where processed metal ions are altered from their

mono- or divalent oxidation states to zero-valent states followed by the nucleation of the reduced metal atoms (Malik et al. 2014). This is instantaneously followed by a growth phase where smaller adjacent particles merge to form thermodynamically stable larger nanoparticles while further biological reduction of metal ions takes place. As growth spurts, nanoparticles aggregate to form diverse morphologies like cubes, spheres, triangles, hexagons, pentagons, rods, and wires (Akhtar et al. 2013). The plant extract's ability to stabilize the nanoparticle determines it's most thermodynamically favorable and stable morphology during the final stage of synthesis. The metal salt concentration, reaction time, reaction solution pH, and temperature of plant extracts drastically influence the quality, size, and morphology of the synthesized nanoparticles (Dwivedi and Gopal 2010; Mittal et al. 2013).

Kesarwani and Gupta (2013) had reviewed several studies on nanostructured systems to optimize the properties of plant extracts. Bhattacharya and Ghosh (2009) devised and used lipid-based systems with green tea and ginseng (*Panax ginseng* CA Meyer) (Araliaceae) extracts, in various formulations, to optimize the absorption of the active components. The use of herbal extracts to synthesize gold and silver nanoparticle was reported by Gardea-Torresdey et al. (2002). Plant extracts contain polyphenols which act as reducing agents whose side chains (mostly –OH group) help in capping and stabilizing nanoparticles and are thus used in the synthesis of metal nanoparticles in large scale.

Gold nanoparticles are extensively used in nanotechnology due to their biocompatibility but are biologically inert; to solve this, they can be engineered to possess chemical or photothermal functionality. This alteration of gold nanoparticle can be done by changing its surface chemistry. Geetha et al. (2013) synthesized gold nanoparticles using the flower of *Couroupita guianensis* tree by a rapid, cost-effective, one-step process. Gold nanoparticles were synthesized without using any stabilizing agent from glucoxylans isolated from the seeds of *Mimosa pudica* (Iram et al. 2014).

Suman et al. (2014) produced gold nanoparticles from aqueous root extract of *Morinda citrifolia.* Arunachalam and Annamalai (2013) using the reducing property of aqueous leaf extract of *Chrysopogon zizanioides* biosynthesized gold nanoparticles with antibacterial, antioxidant, and cytotoxic properties. Very few studies have been conducted on the environmentally benign synthesis of zinc nanoparticle. Nagarajan et al. (2013) made zinc oxide nanoparticle using green *Caulerpa peltata*, red *Hypnea valencia*, and brown *Sargassum myriocystum* which are marine seaweeds. FT-IR spectrometry showed the contribution of fucoidan pigment in the synthesis of nanoparticle.

Rajiv et al. (2013) reported the synthesis of zinc oxide nanoparticle from the leaf extract of the weed, *Parthenium hysterophorus* L. which displayed the size-dependent antifungal activity against *Aspergillus flavus* and *Aspergillus niger.* Mixed copper and zinc oxide nanoparticle were synthesized using *Brassica juncea* L. plant, and it was found that the ZnO nanoparticles were nonuniform in shape (Qu et al. 2012). Sankar et al. (2014) synthesized a rod-shaped CuO nanoparticle using *Carica papaya* leaf extract. Padil and Černík (2013) used gum karaya, a natural nontoxic hydrocolloid, to formulate CuO nanoparticles. Harne et al. (2012)

synthesized copper nanoparticles with excellent long-term stability using the aqueous extract of latex of *Calotropis procera* L.

4.8.4 Synthesis of Metallic NPs From Plants

Lately, plant-derived nanomaterials have drawn intrigue in many fields due to their diverse physicochemical properties. The various parts of plant such as the stem, root, fruit, seed, callus, peel, leaves, and flower are being utilized to synthesize metallic nanoparticles like gold, silver, platinum, zinc, copper, titanium oxide, magnetite, and nickel in various shapes and sizes by biological approaches. The biosynthesis reaction can be modified to fit a wide range of metal concentration and amount of plant extract in the reaction medium; it may transform the shape and size of the nanoparticles (Chandran et al. 2006; Dubey et al. 2010).

4.8.5 Stem as Source for Nanoparticle Synthesis

Shameli et al. (2012) synthesized silver nanoparticles using *Callicarpa maingayi* stem methanolic extract and formed [Ag (*Callicarpa maingayi*)] + complex. Aldehyde groups present in the plant extract are involved in the reduction of silver ions into metallic Ag nanoparticles. The phytosynthesis of silver nanoparticles using *Cissus quadrangularis* extracts at room temperature was reported by Vanaja et al. (2013). Diverse functional groups, like carboxyl, amine, and phenolic compounds, were involved in the reduction of silver ions extracted from the stem part of plant extract. Thus, synthesized silver nanoparticles revealed a good antibacterial activity against the pathogenic bacteria, viz., *Klebsiella planticola* and *Bacillus subtilis*.

4.8.6 Leaves-Mediated Synthesis of NPs

Many plant leaf extracts including *Centella asiatica*, *Murraya koenigii*, and *Alternanthera sessilis* have been investigated as mediators for the synthesis of nanoparticles. AgNPs form potent medicines in cancer treatment and other dreadful diseases. Leaf extracts of *P. nigrum* having longumine and piper longminine are utilized as capping agents for the synthesis of silver nanoparticles and may augment the cytotoxic effects on tumor cells (Jacob et al. 2012).

Vijayakumar et al. (2013) described a green synthesis approach for silver nanoparticles with the *Artemisia nilagirica* plant leaf extract. Leaf-mediated nanoparticles in folklore medicine have a potential use as antimicrobial agents in the present and the near future.

4.8.7 Flowers as Source for NPs Production

Noruzi et al. (2011) explored the usage of rose petals for the eco-friendly synthesis of gold nanoparticles. The extract medium contains a surplus of sugars and proteins which are the main sources for the reduction of tetrachloroaurate salt into bulk GNPs. Similarly, *Catharanthus roseus* and *Clitoria ternatea* are used to synthesize metallic nanoparticle with the desired sizes and shapes. Flower-mediated synthesis of nanoparticles have been very useful in controlling harmful pathogenic bacteria. Vankar and Bajpai (2010) used an eco-friendly method to produce gold nanoparticles using the aqueous extract of *Mirabilis jalapa* flowers which acts as reducing agents.

4.9 Use of Nanoparticles as a Therapeutic in Folklore Medicine

Biological entities can be used in the synthesis of nanoparticles in folklore medicine which can deliver new sources of medicines that are cost-effective, stable, nontoxic, eco-friendly, and synthesized using the approach of green chemistry. Although biological entities have been extensively used to produce nanoparticles, bacteria- and fungi-based techniques need a special culture preparation or isolation techniques, while the use of plants offers a straightforward, inexpensive, easily scaled up, clean, nontoxic, and robust procedure that does not need any such special procedures. Nanoparticles with a specific size, shape, and composition can be potentially produced from plant extracts that can be extensively used in the present medical procedures involving nanoparticles like immunoassays fluorescent labeling, targeted drug delivery, and in bandages as antibacterial agents.

Herbal and folklore researchers have established that therapeutic nanoparticles (NPs) are more effective drug delivery systems when compared to the conventional forms of drugs. Nanocarriers transdermal gel (NCTG) was made from optimized nanotransfersomes of diclofenac diethylamine (DDEA) and curcumin (CRM) for a sustained release and targeted effect. Greater absorption of the drug with coadministration of lecithin was achieved due to the nanosize of NCTG, also giving vesicles a hydration gradient, increased permeability, decreased degradation, and clearance by surfactant compared to marketed gel and plain curcumin gel (Chaudhary et al. 2014). Chaudhary et al. (2013) formulated and optimized nanotransfersomes of DDEA and CRM which provided a large surface area with high penetration potential and achieved high bioavailability. A potential treatment scheme for uncreative colitis was through a synthesis of pH-sensitive nanoparticles of curcumin-celecoxib combination (Gugulothu et al. 2014). The preparation of curcumin solid lipid nanoparticles (CRM-SLN) with a high-loading capacity and chemical stability for

the treatment of oral mucosal infections was reported by Hazzah et al. (2015). Dandekar et al. (2010) successfully demonstrated an effective antimalarial action with curcumin-loaded hydrogel nanoparticles of hydroxypropyl methylcellulose (HPMC) and polyvinylpyrrolidone (PVP).

Prabakar et al. (2013) established the biosynthesis of silver nanoparticles (AgNPs) using *Mukia scabrella* leaf extract; it showed noteworthy antimicrobial activity against MDR-GNB nosocomial pathogens. AgNPs from the aqueous leaf extract of *Bauhinia tomentosa* Linn. were produced, and their in vitro anticancer activity was studied by Mukundan et al. (2015). Khatoon et al. (2015) prepared fluorescent AgNPs with a significant fluorescence and antibacterial activity by using *Artemisia annua* leaf extract, and their biocompatibility was confirmed by checking for cytotoxicity against human erythrocytes.

Islam et al. (2015) synthesized gold nanoparticles (AuNPs) from the gall extract of *Pistacia integerrima* which displayed a notable antifungal and antinociceptive activity. *Aegle marmelos* Correa (AMC) was investigated for the phytofabrication of nickel nanoparticles (NiNPs) from the aqueous leaf extract of AMC. NiNPs are very good anti-inflammatory agents and drug carriers (Angajala et al. 2014). Magnetic nanoparticles (MNPs)-based drug delivery approach for the co-delivery of curcumin and temozolomide was found to be capable of provoking great anticancerous activity (Dilnawaz and Sahoo 2013).

Bitencourt et al. (2016) confirmed the in vitro efficacy against the complications of diabetes mellitus (DM), and the in vivo toxicity was assessed by using an aqueous extract of *Syzygium cumini* seed (ASc) and polymeric nanoparticles containing ASc (NPASc) which showed a high in vitro activity and potential inhibitory activity against ox-LDL particles. Solid lipid nanoparticles (SLNPs) can be utilized as carriers for the extract's phenolic compounds. For the synthesis of SLNPs, witepsol and carnauba (WSLNPs and CSLNPs) loaded with medicinal herbs, extracts of savory, and sage were used. WSLNPs was a more appropriate vehicle for herbal extracts, with a greater stability during digestion and a high release percentage of phenolic compounds self-nanoemulsifying in the small intestine (Campos et al. 2015).

Li et al. (2011a, b) successfully synthesized a stable drug delivery system (SNEDDS) formulation with persimmon leaf extract with a noteworthy improvement in solubility, in vitro release, and bioavailability compared with the Naoxinqing tablets. SNEDDS optimized quercetin (QT) formulae presented an optimum shield against liver damage, compared with QT against hepatotoxicity induced by paracetamol.

Zhao et al. (2010) synthesized a SNEDDS from the rhizome of *Curcuma zedoaria* and formulated a Zedoary turmeric oil (ZTO) having better bioavailability and aqueous dispersion. The manufactured ZTO-SNEDDS could serve as a partial lipid phase with double advantages of increasing drug loading and also curbing the requirement of inert oils. A multiunit drug delivery system (MUDDS) was prepared for a Chinese medicine: Niuhuang Xingxiao Wan (NXW) to supplement the drug

bioavailability and effectiveness. The in vivo assay for antitumor activity showed the potential efficacy of NXW-MUDDS compared to NXW (Shi et al. 2015).

Suganya et al. (2011) studied and prepared topical administration anti-inflammatory agents from nanoemulsion and poly lactic-co-glycolic acid (PLGA) NPs by mixing of four prenylated flavanones isolated from *Eysenhardtia platycarpa* leaves. A nanofiber mat of polycaprolactone (PCL)/polyvinylpyrrolidone (PVP) was created from crude bark extract of *Tecomella undulata* and evaluated for their antibacterial activities. The extract loaded with nanofiber mat of PCL/PVP showed wound healing properties and reduced dermal bacterial infections, inhibiting the growth of such bacterial strains. To improve wound healing in a rat model, Yao et al. (2017) successfully incorporated *Centella asiatica* (CA) extract into electrospun membranes. The wound areas covered with electrospun gelatin membranes containing CA (EGC) membranes showed more collagen deposition and more capillaries than the wound areas to which the other treatments were applied demonstrating the potentiality of EGC membranes in wound dressings.

The use of capsaicin for topical treatment in nanoparticle, microemulsion, or nanocapsule form was demonstrated as a treatment/modulation in specific receptor activity in vivo or in vitro to treat chronic or acute pain and neuropathic pain (Bakthavatchatam et al. 2003, 2004).

Kanazawa (2009) synthesized a nanoparticle comprising of 0.1–100% w/w of a blood circulation promoter such as tocopherol derivative, a nicotinic acid derivative (niacin, vitamin B3), *Swertia japonica* extract (Makino), sunflower, tocopherols (vitamin E), olive oil (tocotrienols), palm oil, and a biodegradable polymer-like protein, such as collagen, gelatin, albumin, casein, acid-treated gelatin, ovalbumin, sodium casein, and so on. An α-, β-, γ-, and κ-casein was also used alone or in combination. The protein was then subjected to a cross-linking treatment after the formation of a nanoparticle by using transglutaminase obtained from guinea pig liver, goat, rabbit, or human liver and treated with organic solvents like ethanol, acetone, and isopropanol. This formulation can be used as a transdermal absorbable agent, topical therapeutic agent, oral therapeutic agent, intradermal parenteral injection, subcutaneous parenteral injection, cosmetic, functional food, supplement, or a quasi-drug.

Leighton and Frangakis (2013) established a method for the treatment of herpes simplex virus-induced inflammation by the topical application of a mixture containing an effective dose of antihistamine, with the base composition containing essential extracts of lemon balm (*Melissa officinalis*), calendula flowers (*Calendula officinalis*), green tea gunpowder (*Camellia sinensis*), and green rooibos (*Aspalathus linearis*) as emulsions, nanoparticles, suspensions, and patches. A nanoformulation of *Butea monosperma* extract was prepared to treat/prevent bone disorders like osteoporosis. The formulation was available as a microemulsion, nanoparticle, nanoemulsion, and microparticle with a dose of 0.1–5000 mg (Maurya et al. 2007).

Mousa et al. (2013) put forth a method and composition to prepare nanoformulations of active ingredients like *Lepidium sativum*, green tea extract, pomegranate extract, or other lepidium extracts; calcium; vitamin D; and antioxidants like flavonoids and/or isoflavones, lycopene, and a combination that is used for the treatment

of osteoporosis in animals. The active ingredient is encapsulated within the nanoparticles selected from a group of chitosan nanoparticles, poly(lactic-co-glycolic acid) (PLGA) nanoparticles, chitosan cross-linked to fatty/bile acids, alginate-chitosan nanoparticles, polyvinylpyrrolidone (PVP) hydrogel nanoparticles, and so on. The present composition was meant for oral, topical, injectable, via toothpaste, and in combination, in cases of osteoporosis and bone fracture in animals.

Shen et al. (2012) prepared a polyester nanoparticles unit containing curcumin and pyromellitic anhydride monomer residues, and a polyethylene glycol monomethyl ether side chain, bound to the polymer backbone and also prepared polyester which possessed an antitumor, antioxidant, anti-inflammatory, and antibacterial properties in the form of nanoparticles, colloidal particles, and vesicles. An herbal composition of a therapeutically effective amount of *Scutellaria baicalensis*, *Glycyrrhiza uralensis*, *Ziziphus jujuba*, and *Paeonia lactiflora* with a chemotherapeutic compound used to treat cancer in mammals was developed by Liu et al. (2013).

DiMauro and Codman (2013) developed methylated curcumin-methoxystilbene hybrid molecules, used in treating cancer. Less than 1% of oral curcumin enters the plasma and undergoes intestine-based metabolism and rejection. The small amount of curcumin that enters the bloodstream is quickly metabolized by the kidney and liver. The intranasal administration of a formulation with an effective amount of curcumin to the olfactory mucosa across the cribriform plate and into the brain can be used to treat neurodegenerative diseases like Alzheimer's disease.

4.10 Advantages and Disadvantages of Nanoparticles From Herbal Extracts in Folklore Medicine

Nanoparticles synthesized using plant extracts are advantageous to the conventional folklore methods of synthesis due to their low cost of production, fewer accidents, and safer products with less waste. All these characteristics make biosynthesis an almost ideal method for making nanoparticles. However, the change in the physicochemical and structural properties of nanomaterials with a decrease in size could be responsible for a number of material interactions that could lead to toxicological effects. These attributes have to be considered when developing a method of synthesis of nanoparticles and formulating nanodrugs.

4.11 Conclusion

In recent decades, folklore medicines are getting popular and have been getting more consideration because of their potential to treat numerous diseases. However, quite a few problems such as poor solubility, poor bioavailability, low oral absorption, instability, and unpredictable toxicity of herbal medicines limit their use.

In order to overcome such problems, nanoparticles can play a vital role in folklore medicine. Development and synthesis of herbal nanoparticles has become a cutting-edge research in the folklore nanoformulation area. The use of nanoparticles of folklore medicine is gaining popularity and has been getting more consideration because of the fact that nanoparticles can be potentially designed to treat various ailments and diseases. Nanotechnology makes use of nanoparticles that have a high surface area and can reach the targeted site because of its extremely small size. Nanotechnology and folklore can be integrated to overcome the limitations of herbal drugs and to optimize its biotherapeutic effects.

Nanoparticles, especially metallic nanoparticles, have attracted a substantial interest in diverse fields such as medicine and agriculture. This review summarizes the application of nanoparticles from plants as promising biotherapeutic agents in folklore medicine. However, the massive diversity of biological entities ranging from microorganisms to plants renders the field largely unexplored. The production of nanoparticles from plants to serve as stable drug delivery agents is due to the fact that these particles are nontoxic, cost-effective, easily scaled up, environment-friendly, and synthesized using the bioreduction approach. Plant extracts can be used to produce nanoparticles with a specific size, shape, and composition.

Plant-synthesized nanoparticles can be used in the current medical procedures involving nanoparticles such as fluorescent labeling in immunoassays, targeted delivery of therapeutic drugs, tumor destruction via heating (hyperthermia), and antibacterial agents in bandages. On another front, plant-synthesized nanoparticles also are potential delivery agents of antimicrobial compounds for use as pesticides for agricultural crops. Metallic nanoparticles prepared from the extracts of many medicinal plants have been combined with folklore medicinal preparations to increase the efficacy of the herbal drugs. Despite the environmental advantages of using biological synthesis over the traditional methods, there are some unaddressed issues such as particle size and shape consistency, reproducibility of the synthesis, and knowledge of the process of synthesizing metallic nanoparticles. Therefore, a more thorough investigation is due, to evaluate and understand the actual plant-dependent materials and involved mechanisms. Nanosized drug delivery systems for herbal drugs can potentially enhance the biological activity and overcome problems associated with plant medicines. However, monumental challenges remain on the implementation of clinically viable therapies in this field. Trials for novel methods to control the interactions of nanomaterials within biological systems are one of the current challenges to translating these technologies into therapies.

The use of nanoparticles in folklore medicine can be potentially designed to increase its bioavailability and for treatment of various ailments and diseases. The review has also demonstrated that folklore medicine biosynthesized in the form of nanoparticle not only increases the solubility and bioavailability of poorly soluble herbals but can also improve biotherapeutic activity of novel herbs.

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Chapter 5
Phytonanotechnology for Enhanced Wound Healing Activity

P. Monika and M. N. Chandraprabha

Abstract Wound healing consists of systematic progression of events that helps re-establish the integrity of the damaged tissue. Several natural compounds have been shown to possess anti-inflammatory, antimicrobial and cell-stimulating properties which contribute to accelerated healing process. Although these natural compounds possess a myriad of biological properties and in general less expensive than the modern treatments, their applications are limited due to batch-to-batch variation leading to contradictory clinical results. However, nanotechnology is a promising tool that can overcome the disadvantages of the natural compounds when they are used alone. In this chapter, we have focused majorly on types, uses, applications and mechanism of action of various phyto-nanomaterials in different phases of wound healing. We have also provided the literature reports available on various in vitro and in vivo studies on use of phyto-nanoformulations for wound healing. Available literature indicates that very few phytocompounds have been converted into nanoformulation for wound healing applications and more research needs to be carried out to explore the potential of other phytocompounds known to possess wound healing activity using nanotechnological strategies. Also, different nano-drug delivery systems can be explored for effective delivery of the phytocompounds at the wound site. In the near future, phyto-nanotechnology can be a boon for the clinicians to treat various non-healing chronic wounds.

Keywords Biological properties · Nanotechnology · Phytocompounds · Phytonanoformulation · Wound healing

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_5

5.1 Introduction

A person might get wounded multiple times in his lifetime, but the wounds that persist throughout are of major concern. These non-healing chronic wounds involve high health care, cost burden and patient incompliance that underline the importance of research in this field. Additionally, time is also an important factor in wound management and repair. Wounds can be classified according to various criteria (Robson et al. 2001). Thus, wounds can be clinically categorized as acute and chronic according to their time frame of healing (Velnar et al. 2009). Acute wounds heal within a predictable time period (1–12 weeks depending on the nature of the wound). However, chronic wounds do not heal in a predicted time period, are more susceptible to infection and are considerably more difficult to manage (Parani et al. 2016).

Worldwide, around one billion people are likely to have acute and/or chronic wounds. Process of wounding and wound healing takes place in all tissues and organs of the body that are common to all tissues. Although the process of healing is continuous, it is arbitrarily divided into different phases in order to aid understanding of the physiological processes that are taking place in and around the wounded area (Richardson 2004). The major biological phases, the different cells of the body, mechanism and time factor involved in each phase are illustrated in Fig. 5.1. Wound healing is a complex biological process involving many interdependent and overlapping sequences of physiological actions. It mainly involves four different phases (haemostasis, inflammation, proliferation and remodelling). Haemostatic events occur immediately after injury. The inflammatory phase begins immediately after injury and may continue for up to 20 days. The proliferation phase is characterized by the beginning of angiogenesis, collagen and growth factor deposition and the formation of the extracellular matrix (ECM). The remodelling or

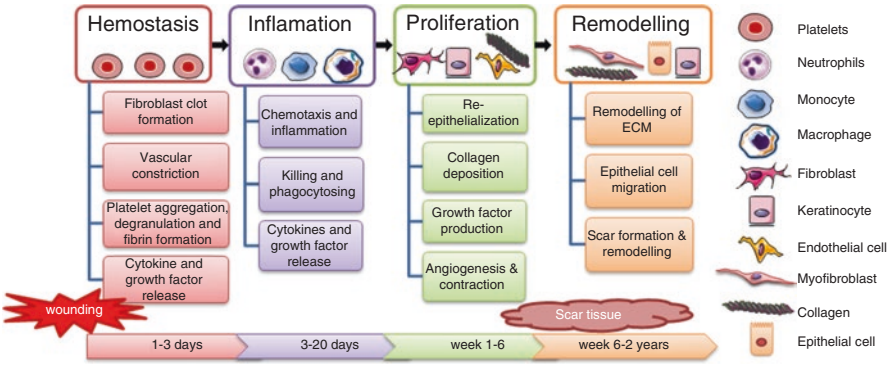

Fig. 5.1 The main biological phases and major mechanism involved in wound healing. The different cells associated and time factor involved in each phase are well defined. The mechanisms underlying the processes described above involve: (1) inflammatory mediators and growth factors; (2) cell-cell and cell-extracellular matrix interactions that govern cell proliferation, migration and differentiation; (3) events involved with epithelialization, fibroplasia and angiogenesis; (4) wound contraction; and (5) remodelling. These mechanisms are initiated at the time of physical injury (wounding) and proceed continuously throughout the repair process (scar formation)

maturation phase usually begins 6 weeks after injury and can take up to 2 years to complete (Garraud et al. 2017). It ends up with the formation of a scar tissue.

5.2 Wound Management

The correct approach to treating wounds should effectively enhance the healing process and should have positive impact on the final clinical outcome. Physiological, endocrine and nutritional support at a clinical level significantly influence repair and, without them, wound healing often fails completely (Attinger et al. 2006). The first stage of wound management should be a thorough assessment of the wound and the patient's past medical history and his/her current medical conditions. The process begins with a diagnosis of the wound's aetiology and continues with optimizing the patient's medical condition, particularly blood flow to the wound area (Attinger and Bulan 2001). The next important step is the lavage of microorganisms, dead tissue and foreign bodies, which can further decrease tissue bacterial count (Dissemond and Goos 2004). An antibacterial solution is usually used for this. Depending on the contamination on the surface, low-pressure or high-pressure irrigation with large quantities of saline is used (Fildes et al. 1991). Thus, wound management involves treating both local and systemic factors associated with the wound.

Various scientific approaches and therapeutic interventions can affect the different processes involved in the cascade of wound healing. Following injury, the healing time may be shorter when there is less injured tissue, for example, during minimally invasive surgery or small cuts or burns, which reduces the amount of soft tissue damage and post-operative morbidity. Novel techniques of topical growth factor application and incisional priming with PDGF or IL-1 can optimize both the cellular and molecular environment, thereby decreasing healing time by modifying the inflammatory phase and accelerating the proliferative phase. Electrical field stimulation may optimize the remodelling phase by promoting more efficient fibroblast recruitment and collagen deposition, prosthetic materials can favour tissue repair and gene therapy, which is currently in preclinical development, may be able to provide a way for selective healing (Velnar et al. 2009). Therefore, it can be concluded that finding novel diagnostic or treatment strategies for wound management at economic costs is indeed a major clinical challenge for both scientists and clinicians.

5.3 Role of Nanotechnology in Wound Healing

Nanotechnology is a rapidly growing and challenging research field worldwide. Nanomaterials have emerged into our daily life, including healthcare and biomedical applications. Numerous novel nanotechnological products for wound healing are currently under investigation. Nanomaterials have attracted considerable attention in research due to their interesting physical and chemical properties which the

micro- or macro-materials do not possess. Comprehensive representation of uses and applications of various nanomaterials in different phases of wound healing is given in Table 5.1. The study of various nanomaterials for wound healing has gained

Table 5.1 Comprehensive representation of uses and applications of various nanomaterials in different phases of wound healing

Type of nanomaterial	Uses and applications in wound healing	Outcome	Phase of wound healing	Reference
Silver nanoparticles (AgNPs)	Antibacterial agent for the treatment of burns, open wounds and several chronic infected wounds	Antimicrobial activity against strains of *B. subtilis*, *E. coli* and *S. aureus* and other skin pathogens	Inflammation	Kim et al. (2007); Ruparelia et al. (2008); Rai et al. (2009); Mosae Selvakumar et al. (2016)
Gold nanoparticles (AuNPs)	Biologically active materials in wound healing and cancer diagnostic agents	Topical application of the product accelerated normal and diabetic wound healing	Proliferation	Chen et al. (2012); Leu et al. (2012)
Copper nanoparticles (CuNPs)	Antibacterial agent for the treatment of diabetic foot-ulcer and burn wound infections	Effective antimicrobial activity against *E. coli* and *S. aureus*	Inflammation	Boateng et al. (2008); Mariselvam et al. (2014)
Titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles	Widely used in the cosmetic and pharmaceutical industry as UV protectors and also as a wound healing material	Formulation of TiO_2 nanoparticles containing *Origanum vulgare* revealed significant wound healing activity in rats	Haemostasis and proliferation	Newman et al. (2009); Sankar et al. (2014)
Tissue-engineered nanofibers	Electrospun nanofibers are suitable for wound dressing, especially for diabetic ulcers and burns. Also used for dermal wound healing	Enables good permeability which protects the wound from bacterial infection; increased rate of wound contraction and epithelialization	Proliferation and remodelling	Khil et al. (2003); Barnes et al. (2007); Chong et al. (2007)
Tissue-engineered nanoparticles	Carbon nanotubes mainly used to develop nanocarriers to promote wound healing	Enhanced wound healing	Remodelling	Bosi et al. (2013)

(continued)

Table 5.1 (continued)

Type of nanomaterial	Uses and applications in wound healing	Outcome	Phase of wound healing	Reference
Nitric oxide (NO) nanoparticles	Used as wound healing agent	Antibacterial activities of the NO designed nanoparticles enhance the wound healing process	Inflammation and proliferation	Schwentker et al. (2002)
Curcumin nanoparticles	PLGA nanoparticles encapsulating curcumin used as a wound healing agent	Showed twofold higher wound healing activity compared to that of PLGA or curcumin	Inflammation, proliferation and remodelling	Chereddy et al. (2013)

special attention because of the unique chemical, physical and biological properties that they exhibit. Due to these multifunctional and beneficial properties, they can further be regarded as therapeutically potential molecules in wound healing. The comparison of different nanoparticles cited in the literature since past 15 years for wound healing studies is given in Fig. 5.2. As per the literature reports, curcumin, a naturally derived phytochemical from turmeric, is most commonly used phytocompound compared to other plant-derived compounds encapsulated into nanomaterials (Fig. 5.2). Some of the commonly used nanomaterials in wound healing studies are discussed in brief below.

5.3.1 Ceramic-Based Nanomaterials

A range of ceramic nanoparticles are investigated for wound healing and infection control. Some of the ceramic nanomaterials include silica, silicate clays, bioglass and zinc oxide nanoparticles. Ceramic nanoparticles are used for controlled delivery of therapeutics due to high surface-to-volume ratio and charged surface. Due to semi-crystalline nature of these nanomaterials, ionic dissolution products of nanomaterials have also shown positive influence on angiogenesis and wound healing (Parani et al. 2016).

5.3.2 Polymeric Nanomaterials

Macromolecular bulk polymers are used as nanoparticle carriers for controlled and sustained release of encapsulated or entrapped therapeutics. The molecular weight of the polymeric nanoparticles can be adjusted as per requirement, and a range of characteristics such as size, shape and hydrophilicity can be modulated. Both

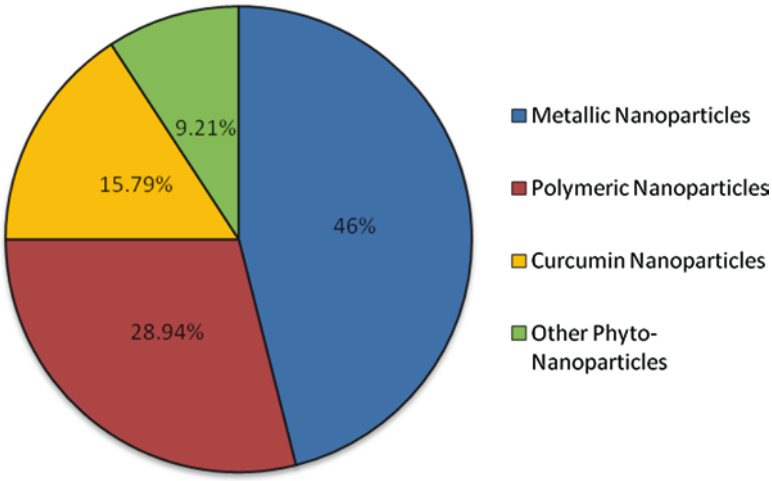

Fig. 5.2 Percentage utilization of different nanoparticles used in the past 15 years for wound healing studies as calculated from the published research articles. In the figure, other phyto-nanoparticles include nanoparticles from *Adhatoda vasica*, *Annona squamosa*, *Azadirachta indica*, *Helianthus annuus*, *Calendula officinalis*, *Olea europaea*, *Withania somnifera*, *Coriandrum sativum*, *Camellia sinensis* and *Aloe vera* which are commonly used plants for wound healing applications

natural and synthetic polymers are investigated for infection control and wound healing applications due to their respective unique physiochemical and biological characteristics. Polymeric nanoparticles are normally bigger than metallic or ceramic nanoparticles (Parani et al. 2016).

5.4 Phyto-Nanoformulations in Wound Healing: In Vitro Studies

Chronic wounds are often complicated by bacterial infection and excess of wound exudates and also characterized by high number of inflammatory cells contributing to morbidity and mortality. Agents commonly used to treat chronic wounds are limited by toxicity, incomplete microbial coverage, inadequate penetration, rising antimicrobial resistance and inability to enhance proliferative phase. Some of the drugs used to treat such wounds also face limitation in wound healing applications due to their physico-chemical properties and their pharmacokinetic profiles. Varieties of plants known to have wound healing activity have been investigated for further applications to explore its potential benefits. Since ancient times, many plant-derived compounds known to possess wound healing properties have been practiced to treat small and deep surface wounds. With the current knowledge on advantages and applications of nanotechnology, some of these plants have been converted into nanoformulation and studied for its wound healing properties under in vitro

conditions. Herbal compounds converted into nanoformulation for wound healing applications are listed in Table 5.2 and currently used phyto-nanomaterials for wound healing is illustrated in Fig. 5.3. Some of the in vitro wound healing studies using phyto-nanoformulation are described below.

Table 5.2 Literature reports on commonly used plants in wound repair therapies and their possible conversion into nanoformulation

Plant name	Part of the plant used	Mechanism of action in wound healing	Nanoformulation of phytocompound	Reference
Adhatoda vasica, Vasaka	Leaves	Enhances fibroplasias and collagen synthesis by enhancing the synthesis of hydroxyproline	–	Zama et al. (1991)
Annona squamosa Linn., Sitaphal	Leaves, powder of seeds	Enhances levels of hydroxyproline, hexosamine, zinc, copper, collagen and elastin in wound, which helps in wound healing	–	Pal (1981); Roopan et al. (2013)
Azadirachta indica, neem	Leaves, bark and fruits	Antiseptic, astringent, insecticidal, larvicidal and anti-inflammatory	–	Bhattarai (1992); Shukla et al. (2002)
Curcuma longa Linn., turmeric	Rhizome	Contains high amount of vitamin A and proteins, which help in fibroblast proliferation and collagen synthesis and thus enhances fibrogenesis and angiogenesis	Curcumin encapsulated nanoparticles; curcumin-loaded PLGA nanoparticles; nanocomposite hydrogel composed of curcumin	Mandal and Chauhan (2000); Tugnaiyat et al. (2000); Li et al. (2012a, b); Chereddy et al. (2013); Krausz et al. (2015)
Helianthus annuus, sunflower	Seed	Antibacterial, anti-inflammatory and healing effects	–	Tugnaiyat et al. (2000)
Calendula officinalis, Marigold	Flower	Stimulates proliferation and migration of fibroblasts in in vitro condition	Solid lipid nanoparticles, gold nanoparticles encapsulated with calendula extracts	Fronza et al. 2009; Demir et al. (2014); Arana et al. (2015)
Olea europaea Linn., olive	Fruit	Causes faster epithelialization, wound contraction and early suppression of inflammation and thus helps in wound healing	–	Jaiswal et al. (2004)

(continued)

Table 5.2 (continued)

Plant name	Part of the plant used	Mechanism of action in wound healing	Nanoformulation of phytocompound	Reference
Withania somnifera, Ashwagandha	Roots	Reduces local inflammation and thus helps in wound healing	–	Sahni and Srivastava (1993)
Coriandrum sativum Linn., Dhania	Leaves and seeds	It has astringent and aphrodisiac action which activates release of sex hormones resulting into accelerated healing process	–	Jaiswal et al. (2004)
Camellia sinensis	Leaves (polyphenols)	It increases collagen synthesis and enhances angiogenesis	–	Hajiaghaalipour et al. (2013)
Aloe vera	Leaves	*A. vera* provides the moist environment to wound surface and increases the collagen content of the wound	Development of novel wound dressings with *Aloe vera*	Anjum et al. (2016)

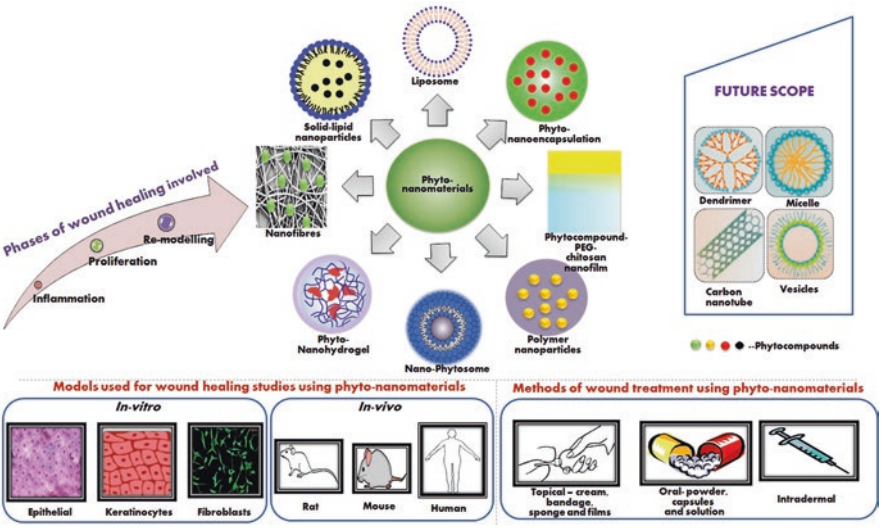

Fig. 5.3 Phyto-nanomaterials currently in use for promoting skin wound healing. This illustration shows various nanomaterials encapsulated with phytocompounds cited in the literature that is used for wound healing applications (top centre). These phyto-nanomaterials are used in various phases of wound healing such as inflammation, proliferation and remodelling (top left). Currently both in vitro and in vivo model systems are used for wound healing studies (below left). Routes of administering the phyto-nanomaterial include topical, oral and intradermal methods, of which topical is the most commonly used method (below right). This figure also represents different nanomaterials that can be used in near future to deliver phytocompounds for wound repair (top right)

Krausz et al. (2015) have explored the benificial properties of curcumin's antimicrobial and wound healing properties. In this study, they synthesized and characterized curcumin nanoparticles (curc-np), which inhibited in vitro growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* in dose-dependent fashion, and inhibited MRSA growth and enhanced wound healing. This nanoformulation overcame the poor aqueous solubility and rapid degradation profile of curcumin. Thus, curc-np may represent a novel topical antimicrobial and wound healing adjuvant for infected burn wounds and other cutaneous injuries.

An in situ injectable nanocomposite hydrogel composed of curcumin, N,O-carboxymethyl chitosan and oxidized alginate as a novel wound dressing was developed for the dermal wound repair application by Li et al. (2012a, b). Nano-curcumin with improved stability and similar antioxidant efficiency compared with that of unmodified curcumin was developed by using methoxy poly(ethylene glycol)-b-poly(ε-caprolactone) copolymer (MPEG-PCL) as carrier followed by incorporating into the N,O-carboxymethyl chitosan/oxidized alginate hydrogel (CCS-OA hydrogel). In vitro release study revealed that the encapsulated nano-curcumin was slowly released from CCS-OA hydrogel with diffusion-controllable manner at initial phase followed by the corrosion manner of hydrogel at terminal phase. Thus, the developed nanophytohydrogel showed an extended and controlled release of curcumin under in vitro conditions.

Few studies have evaluated the wound healing property of curcumin nanoformulation in the form of a film. In the study conducted by Li et al. (2012a, b), curcumin nanoformulation-loaded methoxy poly(ethylene glycol)-graft-chitosan film (curcumin-MPEG-chitosan film) was developed and its applicability in wound healing was investigated. In vitro cytotoxicity test demonstrated that the developed MPEG-chitosan film was non-cytotoxic. Antioxidant efficiency tests revealed that the antioxidant efficiency of curcumin in the film did not show any significant difference compared with that of unmodified curcumin. Thus, a novel curcumin nanoformulation-loaded MPEG-CS composite film was developed in absence of any stabilizer and surfactant.

Similar to nanofilms used for encapsulation of curcumin, there are few studies reporting wound healing property of curcumin loaded into different polymeric nanofibres. Of these in vitro studies available, Merrell et al. (2009) demonstrated the antioxidant and anti-inflammatory properties of curcumin-loaded PCL nanofibre matrix as a potential wound dressing agent. They investigated the feasibility and potential of poly(caprolactone) (PCL) nanofibres as a delivery vehicle for curcumin using human foreskin fibroblast cells (HFF-1). Curcumin-loaded PCL nanofibres (bead-free) were developed using electrospinning parameters. The curcumin was bioactive and fibres showed sustained release of the phytochemical for 72 h and could deliver a dose much lower than the reported cytotoxic concentration. HFF-1 cells showed more than 70% viability on curcumin-loaded nanofibres. The curcumin-loaded nanofibres also exhibited anti-inflammatory activity, as evidenced by low levels of interleukin-6 release from mouse monocyte-macrophages seeded onto the fibres. These results demonstrate the potential of curcumin-loaded PCL nanofibre as antioxidant and anti-inflammatory wound dressing material.

Topical applications of antioxidant agents apart from curcumin, in cutaneous wounds, have attracted much attention. Some of the metallic compounds such as gold nanoparticles (AuNPs), polyphenolic compounds such as epigallocatechin gallate (EGCG) and α-lipoic acid (ALA) were shown to have antioxidative effects and could be helpful in wound healing. In the study conducted by Leu et al. (2012), topically applied gold nanoparticles with epigallocatechin gallate and alpha-lipoic acid were studied regarding their effects on Hs68 and HaCaT cell proliferation . The mixture of EGCG + ALA (EA) and AuNPs + EGCG + ALA (AuEA) significantly increased proliferation and migration of Hs68 and HaCaT cells. Thus, topical application of antioxidant agents helps in wound repair by enhancing proliferation and migration of crucial cells during proliferative phase.

Currently, solid lipid nanoparticles (SLN) composed of various ingredients and loaded with phytocompounds are gaining huge interest in wound healing studies. Arana et al. (2015) prepared SLN composed of long-chain fatty acids (palmitic acid, stearic acid or arachidic acid), Epikuron 200 (purified phosphatidylcholine) and bile salts (cholate, taurocholate or taurodeoxycholate by dilution of a microemulsion). A total of five different systems were prepared and characterized by various spectroscopic, microscopic and calorimetric techniques. The SLN formulation showing optimal properties (lowest size and polydispersity index and highest zeta potential) was obtained with stearic acid and taurodeoxycholate as co-surfactant. A natural compound, *Calendula officinalis*, was extracted and formulated to make an ophthalmic solution. This formulation has anti-inflammatory, emollient and wound repairing activity. *Calendula*-loaded SLN preparations were characterized in order to determine loading capacity and entrapment efficiency. In vitro cytotoxicity and wound healing efficacy of *Calendula*-loaded SLN compared to that of a free plant extract were evaluated on a conjunctival epithelium cell line WKD. These results suggest that the prepared SLN formulation is a safe and solvent-free *Calendula* extract delivery system which could provide a controlled therapeutic alternative for reducing disease-related symptoms and improving epithelium repair in ocular surface.

5.5 Phyto-Nanoformulations in Wound Healing: In Vivo Studies

Curcumin, a well-known topical wound healing agent of plant origin, can be used to treat both normal and diabetic-impaired wounds. Currently, it is the most widely studied phytocompound for wound healing applications. Despite its effectiveness, nano-curcumin dermal delivery is handicapped by hydrophobicity, high metabolism and poor skin permeation. There are few studies reporting use of various polymers encapsulating these nano-phytocompounds, thereby overcoming the disadvantages of low bioavailability of curcumin under in vivo conditions. Some of the in vivo studies on nano-phytoformulations for wound healing are discussed below.

One of the most common polymers, poly(lactic-co-glycolic acid) (PLGA), is used in wound healing processes since the exogenous lactate released from PLGA polymer accelerates angiogenesis. Chereddy et al. (2013) combined the advantages of both PLGA nanoparticles and curcumin and made a nanoformulation of PLGA that encapsulated curcumin. This phyto-nanoformulation was applied to full-thickness excisional wound healing mouse model, which showed twofold higher wound healing activity compared to that of PLGA or curcumin alone. PLGA-curcumin nanoparticles showed high re-epithelialization, granulation tissue formation and anti-inflammatory potential using histology and RT-PCR studies. PLGA nanoparticles offered enormous benefits for the encapsulated curcumin such as protection from light degradation, enhanced water solubility and sustained release of curcumin over a period of 8 days. In conclusion, they demonstrated the additive effect of lactic acid from PLGA and encapsulated curcumin for the active healing of wounds.

Nanophytohydrogel developed by Li et al. (2012a, b) was used for in vivo wound healing study on rat dorsal wounds. Histological study revealed that application of nano-curcumin/CCS-OA hydrogel could significantly enhance the re-epithelialization of epidermis and collagen deposition in the wound tissue. DNA, protein and hydroxyproline content in wound tissue indicated that nano-curcumin and CCS-OA hydrogel could significantly enhance wound healing rate. Therefore, all these results suggested that the developed nano-curcumin/CCS-OA hydrogel as a promising wound dressing might have potential application in the wound healing.

Leu et al. (2012) studied the effect of topical AuEA application in mouse cutaneous wound healing. The application accelerated wound healing on mouse skin. Immunoblotting of wound tissue showed appreciable increase of vascular endothelial cell growth factor and angiopoietin-1 protein expression, but it was observed that there was negligible change of angiopoietin-2 or CD31 after a period of 7 days. After AuEA treatment, CD68 protein expression decreased and Cu/Zn superoxide dismutase increased significantly in the wound area. These studies showed that AuEA significantly accelerated mouse cutaneous wound healing through anti-inflammatory and antioxidant effects.

Li et al. (2012a, b) developed curcumin nanoformulation-loaded MPEG-chitosan composite film for wound healing application. In vivo wound healing tests revealed that the rate of wound reduction was greatly elevated with rapid re-epithelialization in curcumin-MPEG-chitosan film group. Masson's trichrome staining and hydroxyproline measurement in the wound tissue also suggested that application of curcumin-MPEG-chitosan film could greatly increase the collagen synthesis compared with that of MPEG-chitosan film treatment. The nanoformulation was efficient in delivering curcumin at the required amount topically and promoted wound healing in a rat model. These results proved the effectiveness of curcumin-MPEG-chitosan film in the application of wound healing.

Merrell et al. (2009) studied the in vivo wound healing capability of the curcumin-loaded PCL nanofibres. The curcumin-loaded PCL nanofibres demonstrated an increased rate of wound closure in a streptozotocin-induced diabetic mice model. These results demonstrate that the curcumin-loaded PCL nanofibre matrix is a

bioactive wound healing agent under in vivo conditions. Ranjbar-Mohammadi et al. (2016) used diabetic rats to study the potential of electrospun curcumin-loaded poly(ε-caprolactone) (PCL)/gum tragacanth (GT) (PCL/GT/Cur) nanofibers for wound healing. These scaffolds were applied in two forms, acellular and cell-seeded, for assessing their capability in healing full-thickness wound on the dorsum of rats. The quantification studies showed significant wound healing as evidenced by fast wound closure, well-formed granulation tissue dominated by fibroblast proliferation, collagen deposition, complete early regenerated epithelial layer and formation of sweat glands and hair follicles. It is well known that curcumin's therapeutic potential is limited due to its poor aqueous solubility and rapid degradation, which results in less amount of curcumin reaching the target site at its lower concentrations. Considering this drawback, El-Refaie et al. (2015) aimed to enhance concentration of curcumin delivery at wound sites. They evaluated the potential of novel self-assembled nanogels, namely, gel-core hyaluosome (GC-HS), that enhanced curcumin delivery to wound sites. It was evidenced that there was enhanced healing rate and decrease in scar formation. Curc-GC-HS were prepared using film hydration technique and evaluated with respect to size, zeta potential (ZP), entrapment efficiency (% EE) and in vitro release. Structure elucidation was performed using light, polarizing and transmission electron microscopy (TEM). Studies on burned wounds were performed using female Sprague Dawley rats. The in vivo wound healing potential of Curc-GC-HS in terms of skin deposition ability and histology were compared to conventional transfersomal gel (Curc-T-Pl gel) and other conventional gels. Burn wound healing study showed that Curc-GC-HS was the only system exhibiting marked improvement at day 7 of treatment. At 11th day, Curc-GC-HS-treated wounds showed almost normal skin with no scar as confirmed by histological analysis. Curc-GC-HS showed fivefold higher skin deposition compared to conventional Curc-T-Pl gel. Therefore, these studies confirmed novel gel-core hyaluosomes are promising nanogels that increase Curc skin penetration and dermal localization by protecting against degradation.

Another study involving nanohydrogels aimed at the development of a composite material for wound dressing. Anjum et al. (2016) developed nanosilver nanohydrogels (nSnH) containing *Aloe vera* and curcumin that promote antimicrobial nature, wound healing and infection control. Nanosilver nanohydrogels were synthesized by nanoemulsion polymerization of methacrylic acid (MAA) followed by subsequent crosslinking and silver reduction under irradiation. Polyvinyl alcohol/polyethylene oxide/carboxymethyl cellulose matrix was used as gel system to blend with nSnH, *A. vera* and curcumin and coated on to the hydrolysed PET fabric to develop antimicrobial dressings. In vivo wound healing studies were carried out over a period of 16 days on full-thickness skin wounds created on Swiss albino mice. Gel/nSnH/Aloe-treated wounds showed fast healing with minimum scarring, as compared to other groups, thus suggesting *A. vera*-based dressings to be the most optimum one. These results suggest that nSnH along with *A. vera*-based dressing material could be promising candidates for wound dressings.

Servat-Medina et al. (2015) investigated chitosan/sodium tripolyphosphate (CS-TPP) nanoparticles for therapeutic delivery in in vivo conditions. Administration

of CS-TPP nanoparticles encapsulated with standardized extract from the leaves of *Arrabidaea chica* showed a ~60% reduction in indomethacin-induced acute lesions on gastric mucosa in rats. A comparative study between free extract and encapsulated extract demonstrated a fourfold reduced amount of encapsulated extract was needed to achieve the equivalent healing response of free extract for ulcerative gastrointestinal lesions in rats. It was observed that high concentration (250 and 500 mg/L) of free extract was found to be cytotoxic, but the encapsulated CS-TPP nanoparticles promoted cell proliferation. It is important to note that long-term efficacy of CS-TPP still needs to be investigated for in vivo delivery of therapeutics.

5.6 Mechanism of Action of Phytoconstituents

Wound healing is a complex but highly regulated process. Healing of all kinds of wounds follows common steps of recovery. In most situations, infections of wounds characterized by microbial colonization especially with pathogenic bacteria are often inescapable. Infected wounds are one of the main causes for non-healing chronic wounds. Therefore, the utmost aim is to restore the host-bacterial balance by ensuring that the wound is cleaned up and antimicrobial agents are used with moisture retentive bandages. At the same time as oxidative stress during the initial healing process is high, the next objective is to use agents that scavenge the excess of reactive oxygen anions generated at the wound site and rationalize their concentration. Other objectives are to stimulate the adjoining tissues in the wound by bioactive phytoconstituents that facilitate the processes of cell proliferation, remodelling and maturation.

The plant kingdom is rich in chemical constituents for mitigating these objectives acting as antimicrobial agents, free radical scavengers and anti-inflammatory agents. Several compounds have been isolated using various techniques from different parts of the plant. These isolated compounds are known to possess either one or all of the above properties necessary for comfortable healing process. As already mentioned, steps involved in wound repair involve interactions of neutrophils, macrophages, fibroblasts and other cells at the wound site. Required numbers and proper functioning of these cells at respective phases, along with deposition of collagens with proper laying out around the wounds, are very crucial. Such complex processes require understanding of multiple interactions with several agents. Concomitantly, formation of new blood vessels through the process of angiogenesis to ensure continuous supply of nutrients and healing supplements also requires detailed understanding. In all these processes, several compounds together or in single, from the plant extracts, would work synergistically to provide the desired effect. Therefore such phytochemicals concentrated and blended in optimal concentrations from multiple sources are expected to do wonders in wound repair therapies. In the future years, to carry out multitasking efforts in wound healing of all kinds of wounds requires knowledge about the properties of the key constituents in the plant (Ghosh and Gaba 2013). This means that the physico-chemical, biological properties should

be thoroughly understood to determine the mechanism of action of the unique phytoconstituent responsible or contributing for wound healing.

Phytochemical studies are in progress to isolate, characterize and identify the specific bioactive compounds in the plant responsible for wound healing activity. Phytochemical screening has revealed the presence of tannins, flavonoids, alkaloids, proteins and other important constituents. Of these, flavonoids have been documented to possess potent antioxidant and free radical scavenging effect, which is believed to be one of the most important components of wound healing. However, tannins also form an important group of phytoconstituents responsible for antimicrobial activity, thus accelerating the healing process (Chaudhari and Mengi 2006).

As discussed earlier, wound healing experiments can be performed under in vitro or in vivo conditions or both. In vitro methods involve keratinocyte assays, fibroblast assays and epithelial cell assays. Whereas in vivo methods involve using animal models (mostly rats or mice). Initially, one of the kinds of the wound such as incision/excision/burn/dead space wounds is created on the test animal before starting the treatment procedure. The phytocompound (after conversion into nanoformulation) is administered to the test group either topically/orally or intradermally (Fig. 5.3). In addition to this, a stepwise procedure involved in wound healing practices is shown in Fig. 5.4.

In general experiments, control group would be present to carry out the wound healing studies. In general, control group wound shows granulation tissue and

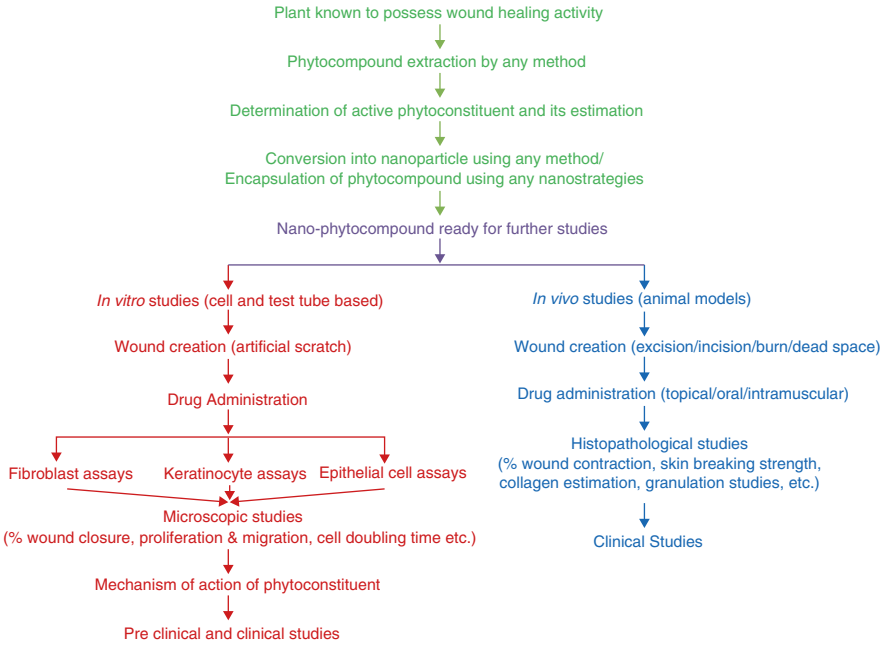

Fig. 5.4 Schematic representation of flowchart describing step-wise practices involved in wound healing studies

fibroblast aggregation. However, studies have shown that animal models treated with plant extracts will show extensive growth of granulation and fibroblast aggregation compared to control. The treated group of wound shows complete healing of wounds with almost normal architecture of the collagen, reticulin (Nayak and Pereira 2006; Shenoy et al. 2009). Increase in tensile strength of treated group wound may be due to increase in collagen concentration that helps in wound contraction, which is facilitated by plant extracts. In excision wound model the increased rate of angiogenesis, wound contraction and decrease in period of epithelialization in the animals treated with plant extracts may be attributed to their broad-spectrum antibacterial activity. This can be studied by the electron microscopic examination which will yield the effect of the extract on angiogenesis, epithelialization or collagen deposition. Significant increase in skin breaking strength, hydroxyproline content and dry granulation tissue is a result of reflection of increased collagen and protein levels, respectively. The breakdown of collagen liberates free hydroxyl proline and its peptides and elevated level of hydroxyl proline is the index of increased collagen turnover.

Therefore, some of the evidences that support enhanced wound healing include free radical scavenging action and the antibacterial property of the phytoconstituents which either due to their individual or additive effect fastens the process of wound healing. The antibacterial action of the phytocompounds on the wound surface may be relied on release of hydrogen peroxide under in vitro and may be due to reduced catalase activity in tissues or blood in in vivo (Cooper et al. 1999). In addition, enhanced wound contraction effect and epithelialization of phytoconstituents could possibly be made use of clinically in healing of chronic wounds. However confirmation of this suggestion will need well-designed clinical evaluation and electron microscopy experiments (Shenoy et al. 2009).

5.7 Conclusion

Chronic wounds represent a major health burden and have a devastating impact on morbidity. It remains a major clinical challenge in long-term care impacting quality of life for patients and healthcare costs. Recent trends move to the development of innovative wound care treatments, combining the use of natural products and nano-strategies. Currently, liposomes, phyto-nanoencapsulation, phyto-nanofibres, nano-phytosome, phyto-hydrogel, phyto-nanofilms, polymeric and solid lipid nanoparticles encapsulating phytocompounds are used for wound healing applications. Nanotechnology-based therapy using natural compounds has recently announced itself as a possible next-generation therapy that is able to advance wound healing to cure chronic wounds. However, to extend the efficacy and the usage of nano-phytocompounds in wound care, multidisciplinary efforts are necessary to prove the safety of these products. It is necessary to investigate their detailed mechanism of action and side effects and develop standard controlled trials. Therefore

with the current knowledge and further progression in research that focus on key issues, phyto-nanotechnology could be a promising approach for enhanced wound healing activity.

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Chapter 6
Chitosan Nanoparticles and Their Applications in Drug Delivery, Hemostasis, and Stem Cell Research

Sanjeev Raghuwanshi, Rashi Agarwal, Ritu Raval, and Ravi Kumar Gutti

Abstract The nanoparticle-based advanced drug formulations present indubitable benefits for drug administration. Over the past two decades, new approaches for the development of novel drug delivery carriers have yielded the opportunities to address and treat many disease conditions. Among the other drug delivery systems, chitosan has recently gained more attention for the development of safe and effective drug delivery systems due to its biocompatibility and unique physicochemical characteristics. Chitosan is a cationic, biodegradable, and biocompatible polymer, which appears to be safe for human dietary use and approved for wound dressing applications. Chitosan has reached a prominent position as a carrier-forming material for the development of polymeric nanoparticles for drug delivery through various routes of administration. Chitosan-based nanoparticles have numerous applications for the treatment of different disease conditions. This chapter mainly explains the characteristics of chitosan, different methods of chitosan nanoparticle preparation, and their applications in drug delivery, hemostasis and stem cell research.

Keywords Chitosan · Chitosan nanoparticles · Hemostasis · Induced pluripotent stem cells (iPSCs) · Oral drug delivery

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_6

6.1 Introduction

The delivery of a drug to the target site is the major question in providing effective treatment for many diseases. The major issues associated with drug delivery are poor stability, water insolubility, low selectivity, high toxicity, and side effects. Good drug carriers play an important role in resolving these issues. Chitosan is not only biologically safe, non-toxic, biocompatible, and biodegradable polysaccharide but also a good drug delivery carrier. Chitosan has gained more attention as a carrier in polymeric nanoparticles (NP) for drug delivery through various routes of administration, and it is because of their better stability, low toxicity, and simple preparation methods. Moreover, because of their small size, they are suitable for mucosal routes of administration, capable of passing through biological barriers in vivo and delivering drugs to the target site. Hence, chitosan nanoparticles have wide development potential as drug carrier (Shi and Fan 2002; Jin and Hu 2008; Mohammed et al. 2017).

Chitosan is a naturally abundant polysaccharide present on the planet. Chitosan is produced by partial deacetylation of chitin through hydration in concentrated alkali (Subramanian et al. 2006). As a natural product and a new drug delivery system, chitosan has attracted increasing attention because of its renewable pharmaceutical adjuvant property and good biocompatibility (Sun and Wan 2007; Tiyaboonchai 2003). In addition, chitosan is a positively charged biomaterial that exhibits absorption enhancing effects (Ravi Kumar 2000). Furthermore, it has been observed that chitosan is soluble in the most common organic acidic solutions at PH less than 6.5, and the physical and chemical properties depend mainly on its molecular weight and degree of deacetylation (Jin and Hu 2008).

Chitosan nanoparticles can be formed by its crosslinking with different agents such as glutaraldehyde (Lin et al. 2007), tripolyphosphate (Mi et al. 2002), and polyaspartic acid sodium salt (Zheng et al. 2007). The methods of preparation of chitosan nanoparticles have been developed more in the last two decades. There are at least four methods available to prepare chitosan-based nanoparticle, which are ionotropic gelation, microemulsion, emulsification solvent diffusion, and polyelectrolyte complex. The most widely studied and frequently used methods for chitosan nanoparticle preparation are ionotropic gelation and self-assembled polyelectrolytes. The simple preparation processes with no use of organic solvent or high shear force are the advantages of these methods. These advantages make these methods applicable to a broad category of drugs including macromolecules of notoriously labile nature. In general, the main factors affecting the nanoparticle formation are the particle size, surface charge, molecular weight of the entrapped drug, and degree of chitosan deacetylation. Moreover, the entrapment efficiency is also affected by the pKa and the solubility of captured drugs. The drug is mostly found to be associated with chitosan via electrostatic interaction, hydrogen bonding, and hydrophobic interaction (Mohammed et al. 2017). In this chapter, we discuss different techniques used to prepare chitosan nanoparticles and their biological applications.

6.2 Characteristics of Chitosan

Chitosan is a derivative of chitin, a natural amino polysaccharide abundantly found in cell walls of fungi, chitin is also a structural element in exoskeletons of arthropods and shells of mollusks (Wang and Xing 2007). N-acetylglucosamine is the monomeric unit of the polymer chitin, the second most abundant carbohydrate after cellulose. Chitin is insoluble in various organic solvents and at neutral pH, which limits its potential for biological applications. Later, a compound called chitosan was derived from chitin by alkaline deacetylation, chitosan, unlike chitin, is soluble in acidic solvents. Naturally, chitosan is found in very less amounts in cell walls of fungi (Muzzarelli et al. 1986). Chitosan is also categorized based on its degree of deacetylation, i.e., the number of deacetylated glucosamine monomers it contains. The solubility of chitosan is because of the amine groups present on it. Chitosan is known to have various biological properties due to its cationic nature, which enables its interaction with negatively charged biological membranes (Divya and Jisha 2018) (Fig. 6.1).

6.3 Methods of Chitosan Nanoparticle Preparation

Nanoparticles are widely famous because of their valuable properties like small size and high ratio of the surface area to the volume. The increased percentage of surface atoms in nanoparticles results in unexpected properties as compared to bulk materials (Gupta et al. 2007). The use of chitosan nanoparticle as a drug delivery system was first reported in 1994 by Ohya et al. (1994). Currently, there are many methods by which chitosan nanoparticles are being prepared such as ionotropic gelation, emulsification solvent diffusion, microemulsion, polyelectrolyte complex, and reverse micellar method. Among these, ionotropic gelation and polyelectrolyte complex are widely used because these methods do not use organic solvents and high shear force in the process (Sailaja et al. 2011).

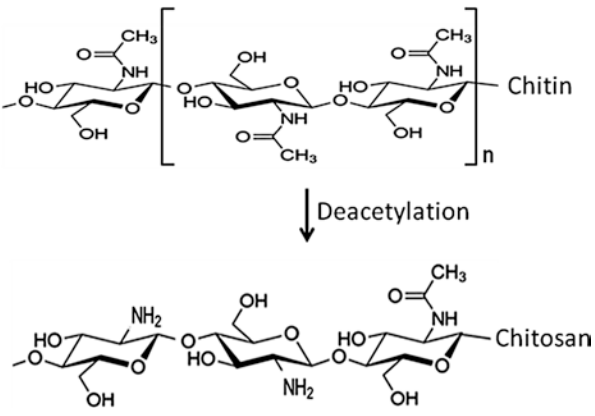

Fig. 6.1 Structures of chitin and chitosan molecules; chitosan is derived from chitin by alkaline deacetylation

6.3.1 Ionotropic Gelation Method

Chitosan nanoparticles prepared by ionotropic gelation method have been examined in past decade for the delivery of various low-molecular-weight drugs (Fernández-Urrusuno et al. 1999; Pati et al. 2011; Morris et al. 2011). Ionotropic gelation involves the interaction between an ionic polymer and a cation or an anion to form a crosslinked structure. Chitosan's amine group makes it a cation polymer that interacts with an anionic polymer. The polyanion most commonly used for the ionic crosslinking of chitosan monomers is the tripolyphosphate (TPP), which is nontoxic. However, to date, the molecular mechanism of nanoparticle formation through ionic gelation is not clear. The chitosan/TPP molar ratio and the molecular interactions are crucial for the nanoparticles mean diameter, stability, and drug release (Zhang et al. 2004; Vyas et al. 2010; Sun et al. 2011).

6.3.2 Emulsification Solvent Diffusion Method

Niwa et al. (1993) developed a method that involves the use of miscible organic solvents into a solution of chitosan. An oil in water emulsion is obtained by mixing organic phase (ethanol or acetone) into the solution of chitosan and stabilizer under continuous stirring, which is followed by the process of high-pressure homogenization. This emulsion is then diluted with large amounts of water for the diffusion of organic solvent which leads to the formation of nanoparticles by polymer precipitation. This method is efficient for entrapping great amounts of hydrophobic drugs. The limitation of this method is the formation of nanoparticles under harsh conditions, such as the use of organic solvent and application of high shear forces (Rajalakshmi et al. 2014).

6.3.3 Microemulsion Method

In this technique, nanoparticles are formed by reverse microemulsion, i.e., water in oil microemulsion, in which the polar groups form a core and hydrophobic groups are outside (Leong and Candau 1982). In this method, chitosan in acetic acid solution and glutaraldehyde (crosslinking agent) are added to a surfactant in an organic solvent such as n-hexane or isooctane. This mixture is continuously stirred overnight at room temperature for complete crosslinking between amine groups of chitosan and glutaraldehyde. This crosslinking in the presence of surfactant results in the formation of nanoparticles, which are then kept for evaporation under low pressure to remove organic solvent. Further, excess surfactant is precipitated with calcium chloride and separated by centrifugation. Finally, nanoparticle suspension is dialyzed and lyophilized to make dry powder (Mitra et al. 2001). This method allows the formation of small size nanoparticles, and the size of nanoparticles can

be controlled by changing the concentration of glutaraldehyde in the preparation process. Some of the limitations of this technique are the use of organic solvents, lengthy protocol, and complex washing process (Maitra et al. 1999).

6.3.4 Polyelectrolyte Complex (PEC) Method

Polyelectrolytes are polymers with a net negative or positive charge at neutral pH. These polyelectrolytes form a complex when there is electrostatic interaction between two oppositely charged polymers. PEC method has been used to prepare chitosan-based nanoparticles for gene delivery applications. DNA is a negatively charged polymer and when it interacts with chitosan, it forms a polyelectrolyte complex due to charge neutralization, which leads to a fall in hydrophilicity. In this method, an anionic solution such as DNA is added to the chitosan dissolved in acetic acid and continuously stirred at room temperature, which is followed by charge neutralization. This is a simple method and does not involve any harsh conditions (Nagpal et al. 2010; Hembram et al. 2014). For drug entrapment, the drug is dissolved in chitosan solution and a polyanion such as alginate is added dropwise for the formation of nanoparticles (Erbacher et al. 1998).

6.3.5 Reverse Micellar Method

Ultrafine nanoparticles with a narrow size distribution can be obtained using this method. First, a surfactant is added to the organic solvent to form reverse micelles. The core of the reverse micelles is used for the formation of nanoparticles since the size of these droplets lies between 1 and 10 nm. Then chitosan solution and drug are added to the surfactant/organic solvent solution. To this, a crosslinking agent is added and kept overnight under continuous stirring. The surfactant is removed by salt precipitation, and the organic solvent is evaporated. This leads to the formation of drug-loaded nanoparticles which is dialyzed using a dialysis membrane and then lyophilized to obtain the powder form (Zhao et al. 2011).

6.4 Applications of Chitosan Nanoparticles in Drug Delivery

6.4.1 Oral, Nasal, and Pulmonary Drug Delivery

There are various routes through which drugs can be administered in our body such as oral, nasal, pulmonary, and ocular routes. The most commonly used route of drug administration is oral because of its convenience (Morishita and Peppas 2006). But, there are several challenges in the oral delivery of protein/peptide and other

sensitive drugs. The stability of a drug is affected by acidic pH of the stomach and digestive enzymes, and they also face mucosal and epithelial absorption barriers; these factors result in low bioavailability of drugs (Mahato et al. 2003). Therefore, chitosan nanoparticles may play an important role in the delivery of acid-labile drugs like proteins and peptides as they protect drugs from pH and enzymatic degradation (Palacio et al. 2016). More interestingly, chitosan nanoparticles are mucoadhesive because of the electrostatic interaction between amine groups and negative charge of mucin which increases the contact time and promotes adsorption (Behrens et al. 2002). These properties enable them to regulate the opening of tight junctions of cell membranes and pass the mucosal epithelial barrier. Moreover, chitosan nanoparticles have greater residence time which improves the drug bioavailability (Artursson et al. 1994). Alginate-chitosan nanoparticles have shown effective delivery of insulin when administered orally in rats (Sarmento et al. 2007). Various formulations of chitosan nanoparticles can be used for various applications in oral drug delivery (Table 6.1). Other mucosal routes through which drugs can be administered are nasal and pulmonary routes. They are considered as better alternatives compared to oral routes because these routes allow rapid and sustained drug delivery with high efficacy and there is no hepatic first-pass effect. Hybrid chitosan-cyclodextrin nanoparticles have shown enhanced transportation of complex molecules across the nasal barrier (Teijeiro-Osorio et al. 2009). The applications of chitosan nanoparticles in pulmonary drug delivery have been discussed in Table 6.2.

Table 6.1 Applications of various chitosan-based therapeutic formulations in oral drug delivery

Drug	Composition	In vivo observations	Reference
Hydrophobic Bay41–4109	LMW chitosan	In vivo studies were performed in rats: The absolute bioavailability of Bay41–4109 NPs was significantly increased, fourfold more than other formulations	Xue et al. (2015)
Insulin	Chitosan, TPP	In vivo studies where decreased glycemia was observed in diabetic rats after insulin NP administration	Diop et al. (2015)
Cyclosporin-A	Chitosan HCl, Poloxamer 188, sodium glycolate, gelatin, soya lecithin	In vivo studies in beagle dogs showed that the relative bioavailability of cy-A was significantly increased by NPs	El-Shabouri (2002)
Enoxaparin	Chitosan, STPP, sodium alginate	The oral bioavailability of enoxaparin in Alg-CS-NPs was higher as compared to enoxaparin solution	Bagre et al. (2013)
Tolbutamide	Chitosan, PLGA, streptozotocin	In adult Sprague-Dawley rats: The TOL-CS-PLGA NPS showed a long-acting hypoglycemic effect over 8 h, longer than metformin tablets	Shi et al. (2018)
Naringenin	Sodium alginate, chitosan, streptozotocin	In vivo study in rats showed that naringenin NPs have better efficacy in lowering blood glucose levels compared to free drug	Maity et al. (2017)

Table 6.2 Applications of various chitosan-based formulations in pulmonary drug delivery

Drug	Composition	In vivo observations	Reference
Heparin (LMWH)	Chitosan, lipoid S100, glycol chitosan	In vivo studies were performed in mice: Lipoid S100-LMWH GCS NPs led to a significant elongation of the coagulation time	Trapani et al. (2013)
Theophylline	Chitosan thioglycolic acid, TPP	In vivo study in mice: Anti-inflammatory effects of theophylline were markedly enhanced when the drug was delivered by TCNs compared to unmodified chitosan or theophylline alone	Lee et al. (2006)
Tetanus toxoid (TT)	LMW chitosan, TPP, trehalose	Intranasal immunization of mice with two doses of TT-CS NPs: The titers were higher for the TT-loaded particles than for the free tetanus toxoid and at post administration IgA levels were significantly higher than the fluid vaccine	Vila et al. (2004)
Estradiol (E2)	Chitosan, methylated β-cyclodextrin, TPP	In vivo study was performed in male Wistar rats: CSF concentration of E2 was significantly increased after intranasal administration of E2-CS-NPs as compared to IV	Wang et al. (2008)
Leuprolide	Thiolated chitosan	In vivo study in male Sprague-Dawley rats showed the improved nasal bioavailability of leuprolide thiolated NPs compared to leuprolide solution alone	Lytting et al. (2008)

6.4.2 Ocular Drug Delivery

Ocular drug delivery is one of the main routes for treating ophthalmic diseases. The main limitations associated with drugs are low residence time and drainage of drugs. Chitosan nanoparticles can be a promising drug delivery system to improve the bioavailability of drugs. It has been observed that chitosan nanoparticles increase cornea residence time of the drug, as they are mucoadhesive. This property is due to the electrostatic interactions between the amine groups and negatively charged mucin (Hirano 1996). After ocular drug administration in rabbits, most of the drug was found to be in the extraocular region, i.e., cornea and conjunctiva, than in the intraocular region, i.e., iris and aqueous humor. This shows that chitosan nanoparticles have great potential in applications at the extraocular level (De Campos et al. 2001). Recently, nanoparticles consisting of hyaluronic acid and chitosan have shown to overcome the corneal epithelial cell barrier by CD44 receptor-mediated endocytotic uptake (de la Fuente et al. 2008).

Chitosan nanoparticles have also been studied in various targeted drug deliveries.

6.4.3 Liver-Targeted Delivery

Chitosan nanoparticles have been studied for the delivery of drugs to liver by the interaction between hepatic receptors and ligand-bearing particulates. Lactosaminated N-succinyl-chitosan nanoparticles were synthesized with a liver-specific drug sodium cyanoborohydride (Kato et al. 2001). On the other hand, in different studies, glycyrrhizin conjugated chitosan nanoparticles have been studied for liver-targeted drug delivery. It was proposed that the binding-sites for glycyrrhizin are present on the surface of hepatocytes, which allow uptake of glycyrrhizin-coated-chitosan nanoparticles by hepatocytes.

6.4.4 Lung-Targeted Delivery

In vitro study on lung cancer cell line (A549 cells) showed that the lung-specific uptake of paclitaxel (anti-cancer chemotherapy drug) was increased by chitosan-modified paclitaxel-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticle. It was observed that under acidic tumor conditions, there are more interactions between chitosan nanoparticles and tumor cells, which lead to an increase in the accumulation of paclitaxel in tumor cells (Trapani et al. 2013).

6.4.5 Cancer-Targeted Drug Delivery

Chemotherapeutics of cancer treatment involves the use of anticancer drugs that have high toxicity. These drugs have low solubility in water, and hence detergents or organic solvents are used to solubilize them. But, this can lead to side effects such as venous irritation and respiratory distress. Hence, nanoparticles are designed for carrying large amounts of drugs and performing controlled site-specific drug delivery to avoid the side effects (Torchilin 2004). A commonly used anticancer drug doxorubicin has adverse side effects such as cardiotoxicity. To reduce these side effects, doxorubicin has been coupled with dextran and encapsulated in chitosan nanoparticles (Mitra et al. 2001). These nanoparticles get accumulated near the tumor site due to their enhanced permeability and retention (EPR) effect. EPR effect increases the accumulation of nanoparticles at tumor site compared to normal tissues. EPR effect of tumor tissues is because of hypervasculature and enhances permeability due to disorganized endothelium and poor lymphatic drainage systems. Hence, researchers are more dedicated toward developing anticancer drugs encapsulated in polymeric nanoparticles, as they are biodegradable and biocompatible (Matsumura and Maeda 1986; Prasad et al. 2017).

6.4.6 Chitosan Nanoparticles in Nonviral Gene Delivery

Viral vectors can efficiently transfer genes but they can cause side effects such as host immune response and pathogenicity. Hence, nonviral systems are being studied as they are safer and lack mutational potential. However, there are various barriers when it comes to gene delivery such as in vivo stability, cell entry, endosome escape, intracellular trafficking, and nuclear entry (Chong and Vile 1996). These barriers can be overcome by using cationic polymers as they can interact with negatively charged surfaces and have adsorption properties as discussed previously. The use of chitosan as a gene delivery system has been tested by many research groups (Guang Liu and De Yao 2002). It was noticed that chitosan has low toxicity compared to other cationic polymers such as poly-L-lysine and polyethylenimine. Transfection using chitosan was successful but its transfection efficiency was less as compared to polyethylenimine. Hence, chitosan nanoparticles were developed to increase transfection efficiency (Krishna Sailaja et al. 2010). The therapeutic use of siRNA in treating various diseases is limited because it is rapidly degraded and has low intracellular association in vitro and in vivo. Chitosan-PG nanoparticles were shown to successfully deliver siRNA in HeLa and HEK 293 FT cells (Lee et al. 2009).

6.4.7 Delivery of Vaccines by Chitosan Nanoparticles

Delivery of vaccines through oral and nasal routes via chitosan nanoparticles is useful for the activation of mucosal protective immune responses. Since nanoparticles are really small in size, this promotes their uptake by M-cells in mucosa-associated lymphoid tissue (MALT). Immunoglobulin A (IgA) is found at the mucosal surface and MALT produces B-cells that express IgA. After activation by vaccine these B-cells migrate to the systemic circulation, where they expand and produce plasma cells expressing IgA, and hence providing systemic immunity (Alonso and Sánchez 2003). There are two mucosal routes though which vaccines can be delivered, oral and nasal. Chitosan nanoparticles are mucoadhesive which enhances the release of vaccines and their residence time. Chitosan nanoparticles containing antigens for influenza, pertussis, and diphtheria were developed. The administration of these vaccines through nasal route showed increased antibody levels in mice (Kreuter 1995). Also, it has been proposed that Pulmonary administration of tuberculosis DNA vaccine may be an effective way compared to intramuscular immunization (Bivas-Benita et al. 2004). Furthermore, a DNA flu vaccine was also formulated in chitosan nanoparticles for nasal administration, which resulted in high antibody level in mice (van der Lubben et al. 2003).

6.5 Applications of Chitosan Nanoparticles in Hemostasis

The chitosan-based hemostatic agents are getting more attention in the management of bleeding. The potential use of chitosan-based nanoparticles as a hemostatic agent has been demonstrated by many research groups. Previously, Yang et al. (2008) observed the aggregation and deformation of rabbit and human erythrocytes when blood incubated with chitosan acetic acid physiological saline solution, but this effect was not observed when solid-state chitosan with a low deacetylation degree was mixed with blood. However, this solid-state chitosan with a low deacetylation degree absorbed more platelets and was more hemostatic. They proposed that solid-state chitosan with a low deacetylation degree could absorb platelets on the surface and that could be a reason for blood to coagulate.

The agglutination in human erythrocytes was also observed with 2% chitosan nanoparticle-based films (Rao and Sharma 1997). Moreover, other reports also recognized that polyphosphate-chitosan formulation in wound dressings increases blood clotting in swine samples (Ong et al. 2008). Moreover, it has been confirmed that chitosan-based nanoparticles promoted agglutination of rabbit blood cells and the agglutination is induced by binding to the erythrocyte membrane (Fan et al. 2012). In other reports, it was observed that pH interferes with hemagglutination process by decreasing erythrocyte binding forces; the agglutination process is more efficient in solutions with pH range which is close to the physiological pH (Barnes 1966). Further, De Lima et al. (2015) demonstrated that ionotropic gelification is an efficient and affordable method for the synthesis of large volumes of chitosan nanoparticles. They observed that the pH neutralization of these nanoparticle solutions results in higher hemagglutination and lower hemolytic activity of human erythrocyte than nanoparticles synthesized under acidic pH. All these reports have pointed to the hemostatic ability of chitosan nanoparticles in solution as well as within nanostructured films. Finally, for the clinical applications, the development of efficient techniques for the production of a large volume of neutral pH, compatible with human blood cells and tissues, chitosan nanoparticles is needed.

6.6 Applications in Stem Cell Research

Chitosan nanoparticles have potential applications in stem cell research. Protein-based reprogramming of somatic cells for the generation of induced pluripotent stem cells (iPSCs) is a nongenetic approach, in which functional proteins such as OCT4, SOX2, KLF4, and c-MYC are delivered to the cell. The technique is safer than transgenic method but the reprogramming efficiency is low due to reduced activity of proteins in soluble systems. Recently, Tammam et al. (2016) have confirmed that encapsulation of OCT4 in nuclear-targeted chitosan nanoparticles strongly stabilized its DNA-binding activity even under cell culture conditions as comparison to soluble OCT4 protein. OCT4-loaded chitosan nanoparticles enabled

the successful delivery of active OCT4 in high concentrations into human fibroblasts. Therefore, chitosan nanoparticles may provide an efficient tool for the generation of transgene-free iPSCs.

In the recent past, accumulating evidence has proved that nanomaterials can facilitate stem cell proliferation and differentiation, researchers have also explored their possible modulating mechanisms in stem cell differentiation. The regulating potential of various chitosan formulations was also tested on stem cells. For example, chitosan-conjugated gold nanoparticles can promote the osteogenic differentiation in human adipose-derived mesenchymal stem cells (hADSCs) through the Wnt/β-catenin signaling pathway (Choi and Song 2015). Chitosan nanoparticles are more preferable as compared to other polymeric nanoparticles because of their biocompatibility and biodegradability. For the delivery of nucleic acid, chitosan nanoparticles were tested in osteogenic differentiation; it was observed that chitosan nanoparticle loaded with hsa-miR-199a-5p agomir can modulate osteogenic differentiation of human mesenchymal stem cells (hMSCs) in vitro and improve bone regeneration in vivo (Chen et al. 2015). Furthermore, hydroxyapatite/chitosan/gelatin 3D porous scaffolds were tested for stem cell differentiation, these biocompatible porous scaffolds increase the proliferation and osteogenic differentiation of human induced pluripotent stem cells (hiPSCs) (Ji et al. 2015). Chitosan and its derivatives show good biocompatibility and batter transfection efficiency among other polymeric compounds. Hence, there are more future possibilities for the development and applications of chitosan nanoparticles in stem cell research.

6.7 Conclusion

The cationic nature of chitosan has led to its applications in biological systems. It helps in protecting the drugs from enzymatic degradation and allow targeted delivery of various anticancer drugs. Their nanometer-size helps in greater absorption and bioavailability. Hence, they can serve as a promising drug delivery system. Herein, we looked at how chitosan nanoparticles are prepared in different methods. Further, we summarized the importance of chitosan nanoparticles in biological applications such as drug delivery, nonviral gene delivery, and delivery of vaccines. We have also discussed current applications of chitosan nanoparticles in hemostasis and stem cell research. Till now, the use of chitosan nanoparticles is being studied at the laboratory level. Additional study would be required before their use as a good drug delivery system.

Acknowledgments This work was supported by Department of Biotechnology (DBT), Department of Science and Technology (DST), and University Grants Commission (UGC) grants of Government of India. We appreciate funding in the form of Council of Scientific and Industrial Research (CSIR) and UGC Fellowships from Government of India.

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Chapter 7
Nanoencapsulation of Anthocyanins for Drug Delivery Systems

José Carlos Andrade, Célia Fortuna Rodrigues, and Natália Martins

Abstract Anthocyanins (ACNs) have a widespread spectrum of biological activities, such as antioxidant, anti-inflammatory, or chemopreventive features, which support human health, although their low bioavailability and extensive biotransformation interfere with these advantages. This chapter underlines the various ACN bioactivities, their bioavailability and stability, as well as the recent nanoencapsulation processes that have improved the ACN effects against different disorders.

Keywords Anthocyanin · Anti-inflammatory · Antioxidant · Bioactivity · Nanoencapsulation · Nanoparticle

7.1 Background

In the last years, a huge attention has been paid to plant-food-derived bioactives for both food and cosmetic industries and more recent for pharmaceutical purposes (Martins et al. 2016a, b). Among these plant-food-derived biomolecules, most often known as phytochemicals, a special emphasis has been given to phenolic compounds, widely recognized for its renowned antioxidant effects, and more recently, notable anti-inflammatory, antitumor, immunomodulatory, and antimicrobial effects have also been listed (Spencer et al. 2012; Carocho and Ferreira 2013; Martins et al.

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_7

2016a). Indeed, since ancient times plant-food-derived preparations have been used for multiple health conditions, not only for preventive but also healing purposes. Nowadays, it is widely recognized that the bioactive effects of these ancient botanical preparations are mostly attributed to its chemical constituents, and thus, more in-depth studies have been performed to improve knowledge on their multiple potentialities and complex interactions.

On the other hand, and not least important to highlight is that, besides an increasing amount of studies have highlighted the multiple potentialities of phytochemicals (e.g., phenolic compounds), it is also true that an intriguing amount of data have underlined some constrains related with its stability, bioavailability, and, consequently, bioefficacy (Fang and Bhandari 2010; Biasutto et al. 2014; Mohan and Nandhakumar 2014; Schön et al. 2018). Among the wide group of phenolic compounds, flavonoids and specifically anthocyanins (ACNs) have been the target of an increasing interest due to its low absorption rates (Norberto et al. 2013), considered as one of the lowest among the flavonoids' class (He et al. 2017). In fact, not only its chemical structure, but also the dose/ingested, the matrix of the food source, and even the food-food, food-drug, and food-nutrient interactions influence its biokinetics (Norberto et al. 2013). In addition, the impact of gut microbiota on phenolic compounds' biotransformation has also been increasingly recognized, leading to the breakdown of the original polyphenolic structures into several low-molecular-weight metabolites, more rapidly absorbed, while others become biologically inactive (Rio et al. 2010; Cardona et al. 2013). In this way, and considering the huge attention paid on ACNs, not only due to its promissory bioactive effects but also intriguing absorption rates, a high amount of studies have been performed to design effective strategies to overcome the ACN biokinetic limitations (Rodriguez-Amaya 2016). Among these strategic techniques, nanoencapsulation, namely, the development of nanoparticle delivery systems, has received a pivotal attention, not only to encapsulate ACNs, but many other bioactive compounds, in order to enhance its gastrointestinal (GI) absorption and even to improve their bioactive potential in the target tissues (Li et al. 2015; Bulatao et al. 2017; He et al. 2017).

In this sense, the present chapter aims to provide a brief overview on drug delivery systems of ACNs, specifically addressing the aspects related with ACN chemical characteristics (such as phytochemistry, bioavailability, and stability) and health-related applications, the currently used techniques for ACN nanoencapsulation, and, lastly, and not least important, an eye-opening look is also given to the key findings reached following the implementation of these techniques.

7.2 Anthocyanins as Source of Biologically Active Molecules

ACNs are natural flavonoid pigments with high antioxidant and anti-inflammatory properties. As a subgroup of flavonoids, ACNs are responsible for the blue, purple, and red color of food (natural colorants). The consumption of fruits, flowers, and leaves rich in these compounds has been related with a reduced risk of several

diseases, such as cardiovascular disease (vasoactive bioactivity), cancer, diabetes, and neurodegenerative diseases (Bonesi et al. 2019). The metabolites might be responsible for these protective effects, although they have been told to have instability at neutral pH and presumed to be related to a significant degradation and subsequent biotransformation (Gowd et al. 2018). In fact, it is reported that dietary anthocyanins undergo a complex metabolism after ingestion and interrelate with endogenous and microbial enzymes. These processes lead to the production of a large number of circulating and excreted anthocyanin metabolites and catabolic products (Tian et al. 2019).

Biotic and abiotic stresses can originate particular defense reactions in plant organs, which after several reactions can produce secondary metabolites and several have been identified. A recent study showed that the application of UV (ultraviolet) stress expressively amplified the expression of GPP synthases, hydroxymethylglutaryl-CoA reductase, deoxy xylose phosphate synthase, 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase, and phenylalanine ammonia lyase genes compared to the control plants. The stress also led to a rise in terpenoids, phenols, flavonoids, anthocyanins, alkaloids, beta-carotene, and lycopene in the analyzed plants (Ghasemi et al. 2019). In another study, four buckwheat varieties (one tartary and three common) allowed the identification of 182 flavonoid metabolites: 53 flavones, 37 flavonols, 32 flavone C-glycosides, 24 flavanones, 18 anthocyanins, 7 isoflavones, 6 flavonolignans, and 5 proanthocyanidins. In the clustering analysis, the authors found alterations in the flavonoid metabolites between tartary buckwheat leaves and common buckwheat leaves, both presenting distinctive metabolites with vital biological functions (Li et al. 2019).

7.2.1 Bioavailability of Anthocyanins

Anthocyanin oral bioavailability has been considered one of the lowest (<1%) among all flavonoid's subgroups (He et al. 2017). However, some recent reports advocate that the real bioavailability of these compounds is underestimated. Anthocyanin bioavailability has been extensively revised recently (Lila et al. 2016; Kay et al. 2017; Braga et al. 2018).

Upon ingestion, ACN disposition follows a unique pattern rather different from those of other flavonoids (Fang 2014). Anthocyanins can be absorbed from the stomach as well as the intestines. Low amounts of unmetabolized parent compounds have been identified in the systemic circulation and urine (Kamiloglu et al. 2015). Stomach transporters, intestinal glucose transporters, and tight junction permeability have all been reported as possible transport mechanisms (Passamonti et al. 2003; Talavera et al. 2003; Oliveira et al. 2015); however, most animal and human studies suggest that ACN absorption takes place mainly in the intestine (Talavera et al. 2003; de Ferrars et al. 2014; Kamiloglu et al. 2015). Like other flavonoids, ACNs are metabolized forming glucurono-, sulfo-, or methyl derivatives in the proximal gastrointestinal tract. Anthocyanins, such as cyanidin-3-glucoside and

pelargonidin-3-glucoside, could be absorbed in their intact form into the gastrointestinal wall, undergo extensive first-pass metabolism, and enter the systemic circulation as metabolites (Fang 2014).

Phenolic acid metabolites were found circulating in the blood in much higher amounts than their parent compounds. These metabolites could be responsible for the health benefits associated with ACNs. Some ACNs can reach the large intestine in significant amounts where they are subjected to microbial catabolism. These metabolites may also contribute to the health effects associated with ACNs. Considering the unmetabolized parent compounds, phase I and phase II metabolites, conjugated products, and microbe-generated metabolites, total bioavailability is much greater than previously believed (Lila et al. 2016). Nevertheless, ACNs are still considered to be generally low and strategies to improve stability and bioavailability are much in need (Mahdavi et al. 2014; Chen and Inbaraj 2019).

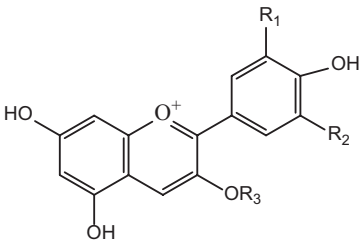
Several fresh reports have explored the ACN bioavailability. Cahyana et al. (2019) identified unmetabolized ACNs and glucuronide derivatives (pelargonidin-3-glucoside was metabolized into pelargonidin monoglucuronide and pelargonidin-3-glucoside monoglucuronide). Delphinidin-3-glucoside was excreted also in the unmetabolized form, significantly and less than polar anthocyanins. Additionally, the mass of urinary ACNs excreted in 24 h was 0.32% of ingested dose of strawberries and 0.22% of ingested dose of red grapes. The peak of anthocyanins' excretion was found in 2–4 h and the proportion of the ingested anthocyanins from the two fruits was not significantly different, yet distinct structures were detected (Cahyana et al. 2019). Likewise, the influence and destination of tart cherry polyphenols in the gut microbiota was assessed by Mayta-Apaza et al. (2018). Tart cherry concentrate juices contained high amounts of ACNs (cyanidin-glycosylrutinoside), flavonoids (quercetin-rutinoside), chlorogenic, and neochlorogenic acids. Gut microorganisms showed to degrade the polyphenols mainly to 4-hydroxyphenylpropionic acids and to minor sums of epicatechin and 4-hydroxybenzoic acids. In this work, in vitro bacteroides increased, possibly due to the input of polysaccharides; however the prebiotic effect was also implied in the *Bifidobacterium* growth from chlorogenic acid. Curiously, in humans high-*Bacteroides* individuals responded with a reduction in *Bacteroides* and *Bifidobacterium* and a proliferation of *Lachnospiraceae*, *Ruminococcus*, and *Collinsella*; low-*Bacteroides* individuals reacted with a rise in *Bacteroides* or *Prevotella* and *Bifidobacterium* and a fall of the other species (Mayta-Apaza et al. 2018). Fang (2014) found that the oral administration of ACNs was found to tail a singular pattern, unlike from other flavonoids, being absorbed from the stomach and intestines. It was shown that active transporters are important in the stomach absorption and in anthocyanins' transfer within the kidney or liver. Particularly, cyanidin-3-glucoside and pelargonidin-3-glucoside were shown to be absorbed in their intact form into the gastrointestinal (GI) wall. Then, there is a first-pass metabolism and finally their metabolites enter the systemic circulation. In fact, some anthocyanins get to the large intestine in high quantities and experience decomposition catalyzed by microbiota, which may contribute to the health effects linked to ACNs in this site. Among the metabolites, the phenolic acids were described in the bloodstream in much superior concentrations than their parent

compounds (Fang 2014). In a different approach, the bioavailability of Delphinol®—a branded standardized maqui berry extract – was explored centered on two selected ACNs. After a supplemented dose in healthy subjects (delphinidin-3-*O*-glucoside (DS) + cyanidin-3-*O*-sambubioside (CS)) and two breakdown products (protocatechuic acid (PCA) + gallic acid (GA)) were assessed for pharmacokinetic parameters. Plasma values of DG and CS showed a quick growth after intake of the extract (DG: 1.0 ± 0.3 h; CS: 2.0 ± 1.1 h) and after 8 h, the concentrations almost returned to baseline levels. Besides, phenolic acids GA and PCA were detected as breakdown products of anthocyanins (Schön et al. 2018). Authors have studied the pharmacokinetics of cyanidin-3-glucoside (C3G) and found that seventeen ^{13}C-labeled compounds were in the serum. Among them, there were (13)C5-C3G, protocatechuic acid, and phloroglucinaldehyde (along with its degradation products). The major phenolic metabolites were hippuric acid and ferulic acid. All identified metabolites displayed dynamic kinetic profiles, having maximal concentrations between 2 and 30 h post-consumption (10–2000 nM) and half-lives amid 0.5–96 h (de Ferrars et al. 2014). Finally, black raspberries (BRB) were shown to be a rich source of ACNs and ellagitannins, yet poorly absorbed and possibly converted into various metabolites by gut microbiota. Their metabolites have demonstrated to impact in the colonic mucosa and to have systemic bioactivity. Gu et al. (2019) have confirmed that a BRB diet influences the colon mucosal microbial composition (luminal microflora). The study revealed that protocatechuic acid was present in higher concentrations in the colon, luminal contents, plasma, liver, and prostate, when compared with urolithins, probably related to the biological activities (Gu et al. 2019).

7.2.2 Stability of Anthocyanins

Owing to their health-promoting properties and potential application as natural colorant, ACNs have been the focus of extensive interest in the food, nutraceutical, and pharmaceutical industries. However, chemical instability of ACNs is one of the major limitations. In fact, ACNs are highly unstable and susceptible to degradation with several factors, including chemical structure, concentration, solvents, pH, temperature, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins, and metallic ions leading to the loss of color, bioactivity, and bioavailability (Cavalcanti et al. 2011; Yousuf et al. 2015; Chen and Inbaraj 2019).

The chemical structure of ACNs (i.e., the number and placement of the hydroxyl (-OH) and methoxyl (-OCH3) groups) (Fig. 7.1) has a significant effect on their stability (Fleschhut et al. 2005). Cyanidin and delphinidin are more stable than malvidin, peonidin, and petunidin due to the blocking reactive OH group by methylation (Riaz et al. 2016). Similarly, ACNs with a 4-substitution are more stable than others. Glycosylation of three positions leads to stability while glycosylation of five positions decreases stability (Riaz et al. 2016). Acylated ACNs were also reported to be more stable than their corresponding non-acylated forms (Sui et al. 2019).



Anthocyanin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH3	H
Petunidin	OH	OCH3
Malvidin	OCH3	OCH3

Fig. 7.1 General structure of most common anthocyanins (flavylium form) found in fruits and vegetables (R3 is usually a sugar moiety)

Usually, ACNs are more stable at low pH values and they degrade at higher pH values (Cavalcanti et al. 2011; Sui et al. 2019). In acidic aqueous solutions ACNs are mainly present as red flavylium cations. The increase of pH (2–4) leads to a loss of the proton producing blue or violet quinoidal base forms (Fig. 7.2). At pH values between five and six colorless carbinol pseudobase and chalcone are formed (Fig. 7.2) (Cavalcanti et al. 2011; Sui et al. 2019). At pH values higher than 7, degradation of ACNs occurs, which depends on their substituents (Castañeda-Ovando et al. 2009). Anthocyanin gastrointestinal absorption is highly affected by this pH dependence (Fernandes et al. 2019).

Temperature is considered as one of the major factors that affect the stability of ACNs (Sui et al. 2019). The stability of ACNs decreases at higher processing and storage temperatures (Jimenez et al. 2010; Jing et al. 2012; Ge et al. 2018). Rise in temperature causes thermal degradation which may result in the formation of brown products, especially in the presence of oxygen (Cavalcanti et al. 2011). Oxygen can also amplify other anthocyanin degradation processes (Cavalcanti et al. 2011). The presence of oxygen can speed up the degradation of ACNs either through direct oxidative mechanism or through the action of oxidizing enzymes such as polyphenol oxidases (Jackman et al. 1987). Ultimately, the instability of ACNs affects their bioaccessibility and further bioavailability.

7.2.3 Phytochemistry, Biological Effects of Anthocyanins, and Health-Related Applications

Though anthocyanins seem to have low bioavailability, their metabolites may have a relevant role in in vivo protective effects. Anthocyanins have shown to interact with the NF-κB and AP-1 signal transduction pathways. These mechanisms not only retort to oxidative signals and arbitrate a proinflammatory effect but also the Nrf2/ARE pathway. Moreover, regulated cytoprotective proteins (e.g., GST, NQO,

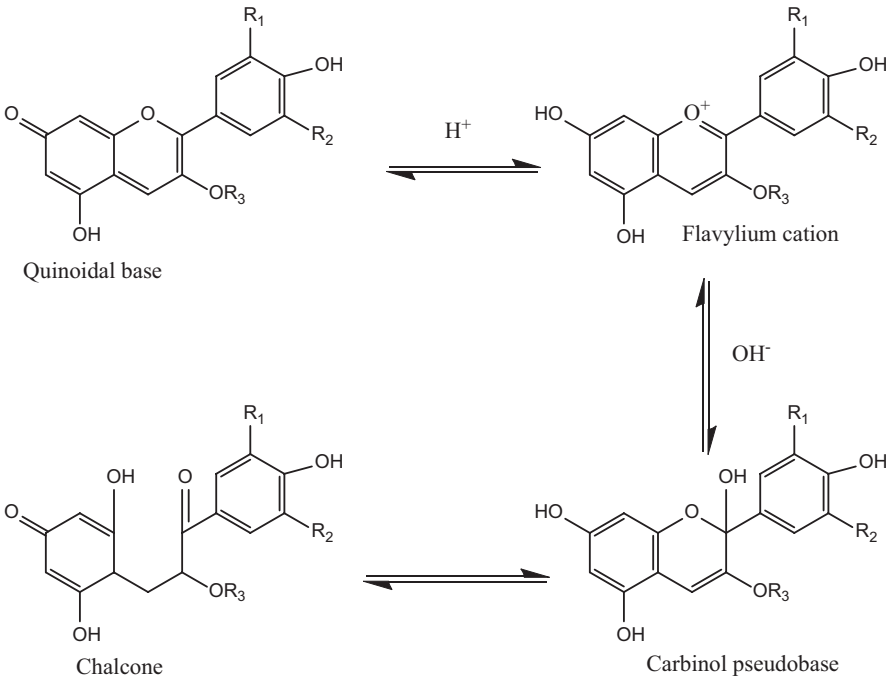


Fig. 7.2 Main equilibrium anthocyanin species and simplified pH-dependent degradation reactions

HO-1) were shown to be directly related in the exclusion or inactivation of toxic compounds and in cellular antioxidant defenses, thus fighting the chemical/oxidative stress. Furthermore, hypothetical crosstalk's could explain the protective effects of these compounds in diverse pathologies, which have distorted balance between these pathways (Speciale et al. 2018). Also, in a recent work, cyanidin-3-arabinoside, cyanidin-3-galactoside, cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, anthocyanin degradation product (4-hydroxybenzaldehyde), or to their gut metabolites (protocatechuic, vanillic, ferulic, and hippuric acid) were studied in order to evaluate the effect of plasma anthocyanins and their metabolites on the adhesion of monocytes to TNFα-activated endothelial cells and on the expression of genes encoding cell adhesion molecules. The authors affirmed that the anthocyanins and their metabolites reduced both the adhesion of monocytes to human umbilical vein endothelial cells (HUVECs) and monocyte adhesion. Additionally, the expression of genes encoding E-selectin, ICAM1, and VCAM1 proposed that other molecular targets are involved in this outcome (Krga et al. 2016). Regarding nephroprotective effects (e.g., lack of tubular and periglomerular necrosis, degenerative changes, inflammatory mononuclear infiltrates, or dilatation of tubules), Popović et al. (2019) described an in vivo study in which 15 different anthocyanins from the bilberry extract were used on the acute kidney injury caused by CCl_4 (Popović et al. 2019). Results showed that the pretreatment of the animals poisoned

with CCl_4 with the anthocyanins led to a clear decrease in the pro-oxidative and pro-inflammatory markers with reduced consumption of the antioxidant defense kidney capacity, compared to the controls. A strong antioxidant and anti-inflammatory effect achieved through the stabilization and neutralization of highly reactive and unstable toxic CCl_4 metabolites was related to the effects of anthocyanins (Popović et al. 2019). The effect of anthocyanin on inflammatory response in osteoarthritis (a degenerative joint disease, which is closely related to cartilage degradation) was recently evaluated using three Thai purple rice cultivars. The results of the study revealed that the main anthocyanins (cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G)) could reduce the inhibition of porcine cartilage degradation and the effects were directly related to the high concentration of anthocyanins in the three rice. The main metabolite of anthocyanin (protocatechuic acid (PA)) showed to have chondroprotective potential by stimulating human chondrocytes, decreasing glycosaminoglycans and collagen breakdown in the experimental model, and, thus, exhibiting anti-inflammatory effects by inhibiting NF-κB and ERK/MAPK signaling pathway (Wongwichai et al. 2019). The role of blueberry anthocyanins and circulating metabolites in the enhancements of the vascular function and the potential mechanisms associated were analyzed by Rodriguez-Mateos et al. (2019). Comparing with control drinks, the purified anthocyanins had a dose-dependent progress of endothelial function in healthy humans, as noticed by flow-mediated dilation (FMD). Blueberry intake amplified acute and chronic FMD and dropped 24-h ambulatory systolic blood pressure both in humans and mice. Besides, it was observed a growth in the expression of 608 genes and 3 microRNAs, which was related to the rise in peripheral blood mononuclear cells. Shapes of 13 metabolites were found to be independent predictors of gene expression variations and there was considerably modulated biological process tangled to cell adhesion and differentiation, migration, and immune response (Rodriguez-Mateos et al. 2019). The biological activities of berry anthocyanins are known to be affected by the existent gut microbiota. The microorganisms transform the compounds into bioactive metabolites, and anthocyanins help the growth of specific bacteria, which clearly signposts a two-way relationship among gut microbiota and anthocyanins. Recently, Petersen et al. (2019) performed an in vivo study that verified if a strawberry supplementation changes gut microbial ecology. Variances in 45 predictive metagenomic functions were identified among the tested groups. The authors also established that the microbial composition (strawberry intake declined *Verrucomicrobia* and boosted *Bifidobacterium*) was highly influenced by both host genotype and strawberry consumption. Finally, *Bifidobacterium* showed to play a pivotal role in the metabolism of anthocyanins (Petersen et al. 2019). In another approach, the capacity of an anthocyanin-rich fraction (ACN) from blueberry, single anthocyanins (cyanidin, delphinidin, and malvidin-3-glucoside), and related metabolites (protocatechuic, gallic, and syringic acid) to resolve an inflammation-driven adhesion of monocytes (THP-1) on endothelial cell (HUVECs) and secretion of cell adhesion molecules E-selectin and vascular cell adhesion molecule 1 (VCAM-1) was determined. The authors reported that ACNs and metabolites appear to resolve, in a dose-dependent manner, THP-1 to HUVECs by declining E-selectin concentrations. Also,

malvidin-3-glucoside showed activity at physiological concentrations (Del Bo' et al. 2019).

Iridoids and anthocyanins from the cornelian cherry fruit have shown to modulate the L-arginine-ADMA-DDAH pathway. Sozański et al. (2019) evaluated the in vivo effects of the oral administration of iridoid loganic acid and anthocyanins, on L-arginine, its derivatives (ADMA, SDMA), metabolites (DMA, L-citrulline), and the hepatic DDAH activity and its isoform expression in a high-cholesterol diet. The two compounds increased l-arginine level and the L-arginine/ADMA ratio, markedly amplified mRNA expression of eNOS in thoracic aortas, and reversed the blood glutathione level depleted by dietary cholesterol and decreased ADMA, DMA, and L-citrulline. Yet, they were not able to reverse the reduction in the blood GPx level, induced by the cholesterol feeding. Also, anthocyanins augmented the activity of DDAH in the liver and DDAH1 and the DDAH2 expression, while loganic acid enhanced only DDAH1 and in a lesser extent. Lastly, the authors did not notice variances in the serum levels of MDA or SOD among the groups (Sozański et al. 2019).

Gutierrez-Albanchez et al. (2019) studied the capability of *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* to control secondary metabolism in strawberry and raspberry and their extracts to change enzyme activities associated to metabolic syndrome. The results showed that total phenolic and anthocyanin contents were higher in strawberries than in raspberries, despite comparable antioxidant activities. Strawberry extracts had a superior performance in enzymes, excluding in inhibiting α-glucosidase. *Bacillus amyloliquefaciens* stabilized the effects of extracts, and *Pseudomonas fluorescens* changed plant metabolism when more inoculations in both species were done. Their activity developed the effects of raspberry extracts on α-glucosidase, COX1, and COX2 and of strawberry on α-amylase and COX1 (Gutierrez-Albanchez et al. 2019).

The effects of anthocyanins on exercise performance without previous muscle-damaging or metabolically challenging exercise are not completely clarified. Reports have been describing exercise performance effects for blackcurrant, but less obvious for cherry, which indicate that the benefits may be owed to the specific source-dependent anthocyanins. Besides, it was also demonstrated that it is possible that the mechanisms by which anthocyanin intake can boost exercise performance involve effects on metabolic pathways, blood flow, and peripheral muscle fatigue or a combination of all three (Cook and Willems 2019).

In an interesting work, authors have cleared up if 2 weeks of flavonoid supplementation (quercetin, anthocyanins, flavan-3-ols mixture) and a 45-min walking session could improve the translocation of gut-derived phenolics into circulation, in a randomized, double-blinded, placebo-controlled, parallel group design. The results suggested that exercises such as brisk walking or intensive running are associated to a 40% higher translocation of gut-derived phenolics into circulation, which was increased when joined with a two-week period of higher flavonoid intake or chronic training (Nieman et al. 2018). In an in vivo study, the inhibitory effect of the oral administration of blueberry anthocyanin extract (175 mg/kg bw/day) pretreatment on glycidamide (GA) metabolism following acrylamide (AA) (35 mg/kg bw/

day) for consecutive 7 and 14 days was explored. The effects indicated that the pretreatment with blueberry anthocyanin extract could significantly block the epoxidation of AA to GA (and its mercapturic acid metabolite N-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)-cysteine (GAMA3)). Additionally, it reduced the addition of GA DNA adduct N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) in several organs and N-(2-carbamoyl-2-hydroxyethyl)valine (GA-VAL) in red blood cells (Wang et al. 2019).

Notwithstanding the composition of anthocyanins in extracts from *Vitis amurensis* Rupr of "Beibinghong," the compound biotransformation pathways in the human intestinal tract have not been scrutinized so far. In a recent report, the biotransformation and the derived metabolites of these compounds were examined and categorized. The results allowed to identify eight types of anthocyanins which were biotransformed in human intestinal microbiota. The microbiota removed all glucosides to generate aglycones, which were afterward transformed into phenolic acid and aldehydes (Zheng et al. 2019). Gowd et al. (2018) subjected black berries to simulated gastrointestinal digestion and gut microbiota fermentation at different time intervals (0–48 h), in order to assess the variations in bioactive components, its antioxidant, and antidiabetic activities. The authors revealed that the antioxidant activity of gut metabolites of blackberries clearly augmented the glucose consumption and glycogen content and bettered high glucose plus palmitic acid-induced ROS overproduction, mitochondrial membrane collapse, and glutathione depletion in HepG2 cells. These upshots indicate that this fruit could be recommended as a functional food with both antioxidant and antidiabetic activities (Gowd et al. 2018). Wu et al. (2018) analyzed several human and animal studies related to blackberry and the prevention of atherosclerosis. The authors disclosed that the elevated levels of polyphenolic compounds of blackberries were considered major in vitro bioactive compounds and, in in vivo, the blackberries metabolites/catabolites (such as phenolic acids) were declared as having the same anti-atherosclerotic effect (Wu et al. 2018).

Actually, the health effects of anthocyanins were shown to be lost in rats whose gut microbiome has been eliminated with antibiotic treatment – pointing to bacterial metabolites of anthocyanins as the likely protective agents. McCarty and Assanga (2018) reported that, after an oral administration of cyanidin-3-O-glucoside (an anthocyanin), the compound was minimally absorbed and ferulic acid (FA) – one of its primary metabolites – was detected in plasma. FA is a strong antioxidant and phase 2 inducer, having demonstrated high in vitro and in vivo anti-inflammatory effects. Actually, in mice with diet-induced weight gain and metabolic syndrome, FA proved to be very protective. FA is also a precursor for lignan synthesis, broadly dispersed in plant-based whole foods, mostly in conjugated form. Recently, it was reported that FA can target protein MyD88, playing a vital role in pro-inflammatory signaling (toll-like receptors and interleukin-1β). The positive effects of FA can probably be extended to several areas, such as hypothalamic inflammation, neurodegeneration, adipocyte and beta cell function, and vascular, cartilage, and bone integrity, but clinical studies are still required to confirm this (McCarty and Assanga 2018).

7.3 Nanoencapsulation of Anthocyanins

The high instability and low bioavailability of ACNs significantly limit their application in the food and pharmaceutical industries. Encapsulation of ACNs can be used to overcome these limitations during processing and storage. Encapsulation technology is a process of entrapping bioactive compounds using a coating material, which protects them from degradation and provides efficient release and delivery for food and pharmaceutical applications (Nedovic et al. 2011). In the last years several classical microencapsulation techniques have being reported to enhance the stability of ANCs (Mahdavi et al. 2014; Robert and Fredes 2015; Chen and Inbaraj 2019). However, these micro-delivery systems are frequently unstable in the physiological environment owing to their large particle size as well as low zeta potential and encapsulation efficiency (Chen and Inbaraj 2019). Recent advances in nanoscience and nanotechnologies allowed several different approaches for preparing nanoparticles to enhance physicochemical stability, bioavailability, and biological activity through active or passive targeting (Santiago and Castro 2016). Nanoencapsulation involves the formation of active-loaded particles with diameters ranging from 1 to 1000 nm. Compared to microparticles, nanoparticles offer a larger surface area, enhanced bioavailability, and improved controlled release, which enable better precision targeting of the encapsulated materials (Fang and Bhandari 2010). Anthocyanin-loaded nanocarriers can be fabricated through lipid formulations, nanoliposomes, polymeric nanoparticles, and by miscellaneous techniques. Table 7.1 presents the main nanocarriers reported for ACNs.

To preserve ACNs and polyphenolic compounds from red cabbage from degradation, solid lipid nanoparticles (SLNs) capable of encapsulating these hydrophilic bioactives were successfully prepared by Ravanfar et al. (2016). SLNs are particles consisting of a matrix made of solid lipid shell. Compared to nanoemulsions and liposomes, SLNs have some advantages including high encapsulation efficiency (EE), possibility of large-scale production and sterilization, and slower degradation rates (Fathi et al. 2012). The SLNs were prepared by the dilution of water in oil (w/o) microemulsions containing ACNs in aqueous media. Due to their hydrophilic nature, ACNs loading into SLNs are challenging as they tend to partition into the aqueous phase during preparation. After optimization of the formulation parameters, SLNs spherical in shape, smooth surface with a mean particle size of 455 nm, and an entrapment efficiency of 89% were obtained. The short-term stability of ACNs was studied in sodium phosphate-citrate buffer (pH = 7.4) at 25 °C (ambient temperature), 40 °C (accelerated testing temperature), and 50 °C (stress or forced degradation temperature). The results showed that the encapsulated ACNs exhibited better stability than the free ACNs, especially at elevated temperatures. Chi et al. (2019) used nanoliposomes to improve the stability and bioavailability of ACNs. Liposomes are spherical, bilayer structures formed by specific polar lipids dispersed in aqueous phases which can embed hydrophobic as well as hydrophilic compounds (Fathi et al. 2012). After optimization, ACN nanoliposomes presented an average particle size of 53 nm and ACN retention rate of 85.60% over 16 days at

Table 7.1 Selected examples of nanosized carriers for anthocyanins

Anthocyanin's source	Encapsulation method	Encapsulating agent	Main achievements	Reference
Black soybean	Complexation	Chondroitin sulfate	Improved stability at different pH and higher antiproliferative activity	Jeong and Na (2012)
Not known	Thermal processing and electrostatic complexation	Whey protein Isolate and beet pectin	Some loss in the antioxidant activity; no inhibition of color degradation	Arroyo-Maya and McClements (2015)
Red cabbage	Microemulsion dilution method	Palmitic acid and surfactants	Improved stability at pH (7, 4) and temperatures (40 and 50 °C)	Ravanfar et al. (2016)
Not known	Emulsification solvent evaporation technique	PLGA /PEG	Improvement of neuroprotective properties	Amin et al. (2017)
Black rice bran	Ionic pre-gelation and polyelectrolyte complex formation	Chitosan and alginate	High encapsulation efficiency and antioxidant activity	Bulatao et al. (2017)
Açaí berry	Modified double-emulsion solvent extraction/ evaporation	EUDRAGIT® L100, PEG 2000, and polysorbate 80	Improved bioavailability	Dupeyrón et al. (2017)

25 °C. The results showed that the nanoliposome encapsulation improved the stability of ACNs under different temperatures (4 and 25 °C), varying pH (3.0, 5.0, and 7.0), white fluorescent light, and when added to milk. Moreover, ACNs from the nanoliposomes displayed a sustained release and high stability in vitro digestion. This work has shown that the nanoliposomes are potential carriers that can be used to stabilize ACNs during their use as a functional ingredient in the food industry. More information about ACN encapsulation on nanoliposomes (and nanoemulsions) can be found in Chen and Inbaraj (2019).

Bulatao et al. (2017) encapsulated successfully ACNs extracted from black rice bran in chitosan alginate particles obtained by ionic pre-gelation and polyelectrolyte complex formation. The average particle size obtained was in the range of 359–636 nm (the authors classified them as nanoparticles below 1000 nm in size) and achieved EE of 69%. The highest antioxidant capability achieved, as measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, was 38%.

He et al. (2017) investigated the encapsulation of blueberry-derived mixture of ACNs in chitosan hydrochloride (CHC)/carboxymethyl chitosan (CMC) nanoparticles prepared by ionic gelation. The negatively charged CMC and the positively charged CHC are two different water-soluble chitosan derivatives possessing biocompatibility, biodegradability, and nontoxicity. CMC and CHC can form a

polyelectrolyte film that maintain the stability of the nanostructures in the gastrointestinal tract, which may expand the ACN absorption site beyond the duodenum. At the optimized conditions the prepared particles averaged 220 nm with 63% encapsulation efficiency. Compared to nanoencapsulated ACNs, the ACN-loaded chitosan nanoparticles showed a slowed degradation in simulated gastrointestinal fluid. Furthermore, when tested in a model beverage system the stability of the ACN-loaded chitosan nanoparticles was also higher than free ACNs. These results show the potential of ACN-loaded chitosan nanoparticles to carry and stabilize ACNS for food applications. More recently, Ge et al. (2019) also used CHC and CMC combined with β-lactoglobulin (β-Lg) to form polysaccharide-protein double-walled materials (chitosan/β-Lg). β-Lactoglobulin is a major component of whey protein in bovine milk which is extensively used as encapsulated material in functional foods due to its high nutritional value, biodegradability, biocompatibility, and pepsin-resistant ability (Livney 2010). After optimization ACN-loaded chitosan/β-Lg nanocomplexes were obtained with particle size of 92 nm and an EE of 69%. When compared to ACN aqueous solution (nonencapsulated form) and to ACN-loaded CHC/CMC nanocomplexes (encapsulated form), ACN-loaded chitosan/β-Lg nanocomplexes (double-walled encapsulated form) significantly improved stability and bioavailability of ACNs during in vitro digestion. Thus, this polysaccharide-protein delivery system possessed a high ACN encapsulation capacity and relatively superior ACN sustained release property.

Dupeyrón et al. (2017) formulated ACNs (extracted from açaí berries) in Eudragit® L100/polyethylene glycol (PEG) 2000 nanoparticles in order to increase their bioavailability. Encapsulation efficiency (EE) ranged from 38% to 73%. These broad values were attributed to ACN solubility. Morphological analysis and DLS measurements indicated that individual nanoparticles (100 nm) were often clustered (800 nm). A delayed release profile of ACNs was observed for all formulations, which may enhance their poor bioavailability.

However, nanoencapsulation of ACNs is not always beneficial. Arroyo-Maya and McClements (2015) studied the use of nanoparticles of whey protein isolate and beet pectin produced by thermal processing and electrostatic complexation, to protect an anthocyanin-rich extract from chemical degradation. The method used involved mixing the two biopolymers at pH 5.8, heating (90 °C, 5 min) to induce protein nanoparticle formation, and then adjusting the solution to pH 4.0 to promote coating of the protein nanoparticles with pectin. A higher loading efficiency (55%) was found when ACNs were added before rather than after (loading efficiency of 35%) the heating step. Although the encapsulation of ACNs within biopolymer nanoparticles improved their heat stability, a loss in the antioxidant activity of the anthocyanins (compared to nonencapsulated ACNs) was observed. This was attributed to the fact that a thermal processing step was required during particle preparation and that some of the anthocyanins may have bound to biopolymers within the particles. Moreover, color measurements indicated that the encapsulation of ACNs did not inhibit its degradation after ascorbic acid addition. Thus, the nanoparticles

developed in this work were not particularly suitable for encapsulation and protection of ACNs.

Other researchers went beyond the demonstration of increased stability and bioavailability and highlighted the therapeutic potential anthocyanin-loaded nanocarriers. In order to increase the bioavailability and improve the free radical scavenging capabilities of ACNs (unspecified molecules or source), Amin et al. (2017) encapsulated them in biodegradable nanoparticles based on poly(lactide-co-glycolide) (PLGA) and polyethylene glycol (PEG) 2000 used as stabilizer. They obtained spherical-shaped nanoparticles with an encapsulation efficiency of 60%, an average particle size in the range of 120–165 nm and a zeta potential of −12 mV. The in vitro release profile observed was biphasic, with initial burst release of ACNs from nanoparticles, followed by a sustained release. The biological activity and neuroprotective effect of encapsulated ACNs were investigated in SH-SY5Y cell cultures. Nanoencapsulated ACNs were found to be more potent than native bulk ACNs and exhibited anti-amyloid, anti-oxidative, and anti-inflammatory properties and were noncytotoxic to SH-SY5Y cell line. Overall, this work not only confirmed the beneficial potential of ACNs in reducing Alzheimer's disease but also proposed an effective mode to improve the efficiency of ACNs through the use of nanodrug delivery systems.

Jeong and Na (2012) proposed a novel nanocomplex using chondroitin sulfate (CS), a sulfated polysaccharide, for the stabilization of ACNs. Sulfated polysaccharides have a strong negative charge on its surface that could maintain charge interaction with ACNs and a stacking structure even at extremely low concentrations. The CS/ACN nanocomplexes were prepared by a simple process without the use of additional energy or organic solvents. The most stable nanocomplex was obtained at a CS/ACN weight ratio of 10/1 with approximately 300 nm in size and 99% EE. The CS/ACN nanocomplex protected the ACNs from degradation at 37 °C and in phosphate buffer under various pH conditions (namely, pH 9). This protection was attributed to the intermolecular stacking interactions, hydrophobic interactions, and charge-charge interactions. Chondroitin sulfate tightly weaved ACNs together so that the nanocomplex maintained its structure without the dissociation of ACN, even at extremely low ACN concentrations. Moreover, the CS/ACN nanocomplex effectively inhibited the proliferation of cancer cells (HeLa) compared with free ACNs. Later the same research group used these CS/ACN nanocomplexes to develop a reactive oxygen species (ROS) sensitive drug carrier (Jeong et al. 2016). Having observed that CS/ACN nanocomplex had a high ROS scavenging capacity and that it decomposed into water-soluble compounds when it reacted with ROS; the authors assumed that a loaded antitumoral drug could be released via CS/ACN nanocomplex destruction at the ROS-enriched tumor region. Doxorubicin hydrochloride (DOX) was loaded in the CS/ACN nanocomplex (CS/ACN/DOX) via intermolecular stacking interaction using a simple method in aqueous phase. The ROS sensitive drug release of CS/ACN/DOX was confirmed under in vitro physiological conditions. The results demonstrated that 1.67 times higher DOX release occurred in CS/ACN/DOX for 48 h compared to CS/DOX (ACNs absent sample).

Drug release and nanocomplex destruction were induced by ROS-mediated ACN degradation. The ROS sensitive therapeutic potential of CS/ACN/DOX was confirmed in an HCT-116 tumor-bearing animal model. The results indicated that DOX was released from the intravenously injected CS/ACN/DOX in the tumor tissue. Overall, this work put in evidence promising potential of CS/ACN/DOX for improving tumor therapy.

Samadder et al. (2017) loaded pelargonidin (PG) into PLGA nanoparticles for therapeutic management of mitochondrial dysfunction frequently found in diabetic conditions. Pelargonidin is one of the six most abundant anthocyanidins present in many fruits and vegetables. In animal models, PG has shown antigenotoxic effects and curative efficacy against an induced diabetic state (Khandelwal and Abraham 2014; Samadder et al. 2017). The average size of the formulated pelargonidin nanoparticles (nano-pelargonidin, NPG) was around 12 nm. Relative functional efficacy of NPG and pelargonidin was evaluated on L6 skeletal muscle cells, considered suitable for such studies in vitro by ALX (laboratory diabetic inducer) treatment. Nano-pelargonidin produced better effects (even at a nearly tenfold reduced dose) in protecting the cells from glucose imbalance than that of PG alone. This protection was attributed to its smaller size, ability of faster entry, and drug delivery at target-specific sites. Moreover, the polymers being biodegradable, biocompatible, and nontoxic could produce suspended release and without any cytotoxic effects to normal cells. This work showed the potential of PG and especially of NPG in formulations designed to inhibit the progression of diabetic complications associated with mitochondrial dysfunction seen in diabetes.

7.4 Concluding Remarks and Upcoming Perspectives

Anthocyanins are natural color pigments with an important role in antioxidant and anti-inflammatory bioactivities. Food rich in ACNs have been linked to a reduced risk of numerous diseases, such as cancer and diabetes, yet the instability, degradation, and subsequent biotransformation have been an obstacle to the bioactivity. The nanoencapsulation proved a higher protection of the ACNs, allowing a higher absorption and, thus, improved biological effect after consumption.

Acknowledgments C.F.R. would like to thank for the UID/EQU/00511/2019 Project—Laboratory of Process Engineering, Environment, Biotechnology and Energy—LEPABE financed by national funds through FCT/MCTES (PIDDAC). N.M. would like to thank the Portuguese Foundation for Science and Technology (FCT-Portugal) for the Strategic Project ref. UID/BIM/04293/2013 and "NORTE2020 - Northern Regional Operational Program" (NORTE-01-0145-FEDER-000012).

Conflict of interest: The authors declare no conflicts of interest.

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Chapter 8
Nanotechnology in Oral Drug Delivery: Salient Aspects, State of Art, and Applications

Mitali Patel, Garima Joshi, and Krutika K. Sawant

Abstract Nanotechnology has a revolutionary impact on medicine and pharmaceutical sciences. It can play a major role in diagnosis and therapy of cancer, cardiovascular diseases, asthma, hypertension, HIV, diabetes, and infectious diseases. The oral route is associated with the greatest degree of patient compliance (especially for chronic conditions) as it ensures convenience, enables self-administration, and offers great flexibility in dosage regimen. Nanosystems are stable, capable of being functionalized, biocompatible, and directed to specific target sites in the body after systemic administration. Oral products do not require sterile conditions for their manufacture, which reduces production costs. Nanocarriers increase oral bioavailability of drugs due to their specialized uptake mechanisms such as absorptive endocytosis and are able to remain in the blood circulation for a long time, releasing the incorporated drug in a controlled fashion, leading to less plasma fluctuations and minimize side effects. The gastrointestinal (GI) tract offers extensive surface area (300–400 m^2) for drug absorption by absorptive epithelial cells (enterocytes). Nanoscale size nanostructures are able to penetrate tissues and are easily taken up by cells, allowing for efficient delivery of drugs to target sites of action. Uptake of nanostructures has been reported to be 15–250 times greater than that of microparticles in the 1–10 μm range. Various nanotechnology-based drug delivery systems are designed, extensively researched, and explored, viz., liposomes, niosomes, polymeric nanoparticles, and solid lipid nanoparticles. Various designing strategies can be adopted for nanoparticles for different regions of gastrointestinal tract like stomach targeting, small intestine delivery, lymphatic targeting, colon targeting, and

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_8

systemic circulation. In this chapter, we aim to discuss various aspects of nanoparticles for oral delivery, viz., strategies, challenges, design, and applications of nanotechnology in oral delivery of drugs.

Keywords Applications · Bioavailability · Enterocytes · Nanotechnology · Oral delivery · Poor solubility

8.1 Introduction

Oral drug delivery is the choicest and most readily accepted form of drug administration because of its noninvasive nature. It is preferred because of the various advantages over other routes of drug delivery. The oral route presents the advantage of avoiding pain and discomfort associated with injections as well as eliminating contaminations. The other advantages include patient convenience and compliance, which increase the therapeutic efficacy of the drug. Oral formulations are also cheaper to produce because they do not need to be manufactured under sterile conditions (Salama et al. 2006).

Despite these potential advantages, oral formulations face several common problems: (1) poor stability in the gastric environment, (2) poor bioavailability, and (3) the mucus barrier can prevent drug penetration and subsequent absorption. Many drugs are currently used as parenteral formulations because of their poor oral bioavailability. This is due to several unfavorable physicochemical properties, such as large molecular size, susceptibility to enzymatic degradation, poor stability in the gastric pH environment, poor penetration at the intestinal membrane, short plasma half-life, immunogenicity, and tendency to undergo aggregation, adsorption, and denaturation prior to absorption (Yun et al. 2013).

Despite the extensive research and success stories with other routes for drug delivery, the oral route is still the most preferred route because of its basic functionality and the advantages that ensue. Oral delivery is by far the easiest and most convenient way for drug delivery, especially when repeated or routine administration is necessary (Gomez-Orellana 2005). But the challenges associated with oral route include exposure to extreme pH variations, intestinal motility, mucus barrier, P-glycoprotein efflux pump, and impermeability of the epithelium (Hauss 2007). Moreover, the gastrointestinal tract provides a variety of barriers to the delivery of drugs, including proteolytic enzymes in gut lumen and on the brush border membrane, mucus layer, gut flora, and epithelial cell lining.

One of the most attractive areas of research in drug delivery today is the design of nanosystems that are able to deliver drugs to the right place, at appropriate times, and at the right dosage. Nanoparticles are solid colloidal drug carriers ranging from 1 to 1000 nm in size. Nanoparticulate delivery systems have the potential power to improve drug stability, increase the duration of the therapeutic effect, and permit administration through enteral or parenteral administration, which may prevent or

minimize the drug degradation and metabolism as well as cellular p-glycoprotein efflux (Sarmento et al. 2007). Nanoparticles have been extensively studied for per-oral drug delivery, for systemic effect following uptake from enteron, or to act locally in the gastrointestinal tract. Nanoparticles are expected to address the specific issues for drug delivery like low mucosal permeability, absorption windows, low solubility of the drugs, gut metabolism, and first pass effect. The potential advantages of nanoparticles as oral drug carriers are enhancement of bioavailability, delivery of vaccine antigens to the gut-associated lymphoid tissues (GALT), controlled release, and reduction of the gastrointestinal irritation caused by drugs (Hariharan et al. 2006). The nanocarriers can improve the oral bioavailability of poorly bioavailable drugs due to their specialized uptake mechanism by preventing first pass metabolism of encapsulated drugs.

The nanoparticles by virtue of their size and colloidal properties can be targeted to GALT (gut-associated lymphoid tissue) to deliver high loads of drug to lymphatic tissue and then to systemic circulation. The nanoparticles are taken up intact by M cells of Peyer's patches in the intestine-associated lymphoid tissue. M cells lack fully developed microvilli in comparison to the neighboring absorptive cells and deliver the particles taken up to the lymphatics from where they, in a size-dependent manner, are then released into the bloodstream (Caruthers et al. 2007).

This mechanism provides a chance to target cancers of lymphatics as well as targeting antiretroviral drugs to the viral reservoirs. Furthermore, nanoparticles are capable of sustaining drug release in plasma for longer time period, thus reducing frequency of administration. The nanoparticle surface can also be modified to enhance or reduce bioadhesion to target specific cells. Polymeric nanoparticles are of especial interest from the pharmaceutical point of view. First, they are more stable in the gastrointestinal tract than other colloidal carriers, such as liposomes, and can protect encapsulated drugs from gastrointestinal environment. Second, the use of various polymeric materials enable the modulation of physicochemical characteristics (e.g., hydrophobicity, zeta potential), drug release properties (e.g., delayed, prolonged, triggered), and biological behavior (e.g., targeting, bioadhesion, improved cellular uptake) of nanoparticles. Third, their submicron size and their large specific surface area favor their absorption compared to larger carriers. Also, the particle surface can be modified by adsorption or chemical grafting of certain molecules such as poly(ethylene glycol) (PEG), poloxamers, and bioactive molecules (lectins, invasins).

8.2 Oral Route, Barriers, and Challenges

Though oral route is the most common, convenient, and preferred route for drug administration owing to its ease of administration, noninvasiveness, and patient's compliance, there are various pitfalls associated with oral drug delivery (Bhardwaj et al. 2005; Gomez-Orellana 2005). First, the physicochemical properties of the drug molecule such as low aqueous solubility, low permeability, rapid metabolism,

and instability in gastrointestinal tract (GIT) reduce the oral bioavailability (BA). Second, the hydrophilic environment of GIT can restrict the bioavailability of lipophilic drugs. Third, the hepatic first pass metabolism is the major reason of poor BA upon oral administration (Mundada et al. 2016).

With the advent of drug design, thousands of new chemical entities are discovered and synthesized. But most of the newly discovered drugs belong to biopharmaceutical classification system (BCS)—II and IV drugs. These two features, poor aqueous solubility and low permeability, limit the oral bioavailability of drugs. Poor aqueous solubility of drug leads to low dissolution and hence poor absorption from GI mucosa (Stegemann et al. 2007; Yun et al. 2013).

Besides this, low oral bioavailability could be attributed to other factors such as hepatic first pass metabolism, drug efflux by P-glycoprotein, and chemical and enzymatic degradation. Also, the pH of GIT varies from the highly acidic in the stomach to the slightly basic in the intestine.

When a lipophilic drug enters into GIT, various events limit its absorption. First, the limited capacity of the emulsification process by biliary secretions in the upper part of GIT is a crucial step in the solubilization of drug. This process enhances the solubility of drug in the aqueous intestinal milieu by facilitating the formation of minute micelles and reaches to the enterocyte. Second, unstirred water layer (UWL) is a main hydrophilic barrier for the absorption of hydrophobic drugs (Liu et al. 2018).

In addition to these barriers, a biochemical barrier, namely, enterocyte CYP 3A4 (CYP3A4) enzymes, located in the endoplasmic reticulum of the enterocyte, is responsible for most of drug metabolism in the intestinal wall. Also, the drug efflux transporters such as P-gp are also responsible for poor oral bioavailability of various drugs (Kalepu et al. 2013). There is a link between the activity of the CYP3A4 enzymes and the P-gp transporters which prevent the access of lipophilic drugs in the systemic circulation. A drug molecule that is able to get away from the intra-enterocyte metabolism and the P-gp efflux system is transferred to the liver before reaching the systemic circulation, where it is exposed to various metabolic enzymes. This first pass hepatic metabolism is another significant barrier to the systemic bioavailability of lipophilic drugs.

8.2.1 Transport Mechanisms Across Intestinal Epithelium

There are different mechanisms through which molecules cross the cell membrane, viz., paracellular, transcellular, carrier-mediated, and receptor-mediated transport (Fig. 8.1). Absorption mechanism is dependent on different characteristics such as hydrophobicity, molecular weight, ionization constants, and pH. Hence, it is necessary to understand different mechanisms for designing suitable delivery systems for oral delivery of drug (Rahman et al. 2011).

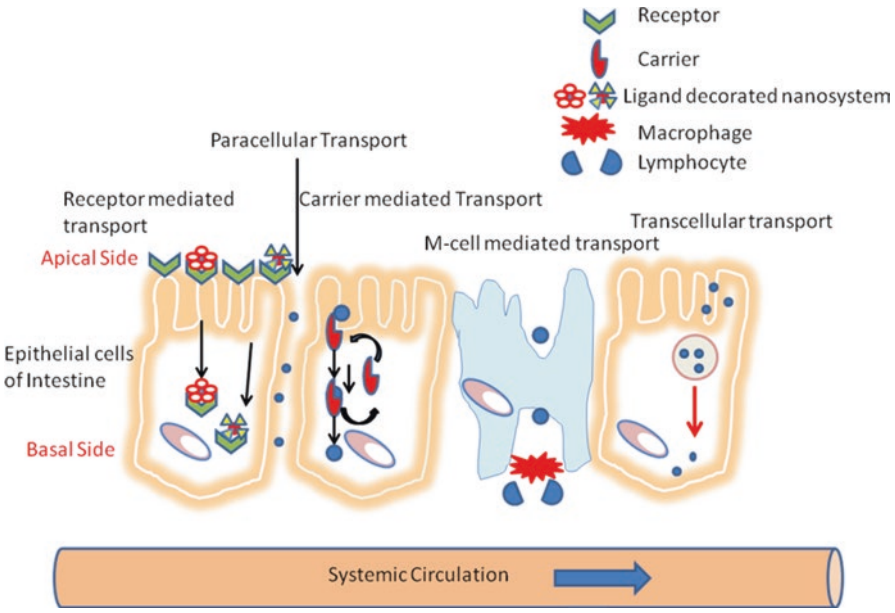

Fig. 8.1 Transport mechanism across intestinal epithelium

8.2.2 Paracellular Transport

Paracellular transport is the pathway of substances across an epithelium i.e. passage through the spaces available between the epithelial cells. This transport results from passive diffusion and under the control of tight junctions. A tight junction is the major rate-limiting barrier toward the paracellular transport of ions and larger substances (Madara 1998; Yun et al. 2013). Paracellular transport varies among epithelia in terms of electrical resistance and ionic selectivity. The tight junction provides biophysical properties with ion channels, including its size and charge selectivity, ion concentration-dependent permeability, competition between permeant molecules, anomalous mole fraction effects, and sensitivity to pH (Pappenheimer 1987; Tomita et al. 1988).

8.2.3 Transcellular Transport

Transcellular transport is defined as the transport through the intestinal epithelial cells by transcytosis. Transcellular transport depends on various factors such as (1) physicochemical properties of particles, (2) the physiology of the GI tract, and (3) the animal model used to study the uptake (Burton et al. 1991; Tang and Goodenough 2003; Shakweh et al. 2004; Florence 2004).

Enterocytes and M cells are mainly responsible for transport. Enterocytes represent the cells lining the gastrointestinal tract and M cells are located within the epithelium of Peyer's patches and represent a very small proportion of the intestinal epithelium (Giannasca et al. 1999). Furthermore, M cells represent a potential portal for oral delivery of proteins and peptides due to their high endocytosis ability. M cells have high transcytotic capacity and transport a wide variety of materials, including nanoparticles (Frey and Neutra 1997; Clark et al. 2000). M cells take up macromolecules and particles by endocytosis mechanism via clathrin-coated pits and vesicles, fluid-phase endocytosis, and phagocytosis (Buda et al. 2005).

8.2.4 Carrier-Mediated Transport

This transport mechanism includes transfer of drugs across the cell membrane or entire cell and then release from the basal surface of the enterocyte into systemic circulation. Small hydrophilic molecules are generally transported via this mechanism. Active absorption needs energy-dependent uptake of specific molecules by carriers. The carriers recognize specific target through membrane receptors present on cells and transfer them across the cell membranes, even against the concentration gradient. For example, monosaccharides, small di−/tripeptides, and amino acids are transported by a carrier-mediated transport process (Yun et al. 2013).

8.2.5 Receptor-Mediated Transport

In receptor-mediated transport process, protein drugs act as either receptor-specific ligand for surface-attached receptors or as a receptor for surface-attached ligands (Russell-Jones 1996). Hence, this receptor-mediated transport can be used to enhance the oral bioavailability of protein drugs by modification such as receptor-specific ligands with peptide and protein drugs. This transportation cell absorbs molecules by inward budding of the plasma membrane, which leads to formation of a vesicle and release at the basal side. This transportation is also known as endocytosis. Endocytosis comprises of phagocytosis, pinocytosis, receptor-mediated endocytosis (clathrin-mediated), and potocytosis (nonclathrin-mediated) (Charman and Porter 1996; Swaan 1998).

8.3 Nanotechnology-Based Formulations for Oral Delivery

Different nanotechnology-based formulations which can be used for oral delivery are shown in Fig. 8.2 and discussed below. The oral administration of nanoparticles transported via the intestinal lymphatics offers advantages, namely, (1) Oral

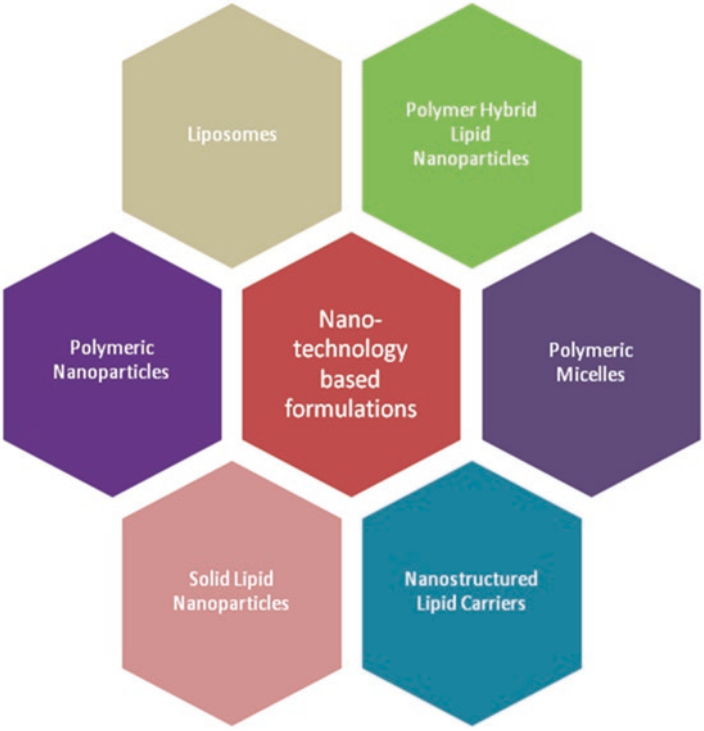

Fig. 8.2 Nanotechnology-based formulations for oral delivery

bioavailability enhancement by reducing hepatic first pass metabolism, (2) target drugs to regions of the lymphatics supplied by mesenteric lymph, and (3) gives sustained drug release which maintains plasma drug concentration for longer time.

8.3.1 Polymeric Nanoparticles

The historical development of PNP was created by Paul Ehrlich with the first experimental efforts by Ursula Scheffel. Extensive works were conducted by the group of Peter Speiser at ETH Zürich in the late 1960s and early 1970s. Nanoparticles can be defined as particles having diameters in the range 10–100 nm are nanoparticles (Moritz and Geszke-Moritz 2015; Prasad et al. 2016).

Polymeric nanoparticle is a rising tool and widely applicable in the field of pharmaceutical development (Prasad et al. 2017). They offer several benefits. First, they are more stable in the GIT than liposomes and protect the drug from gastrointestinal environment. Second, the use of various polymeric materials enables the modulation of physicochemical characteristics (e.g., hydrophobicity, zeta potential), drug release properties (e.g., delayed, prolonged, triggered), and biological behavior

(e.g., targeting, bioadhesion, improved cellular uptake) of nanoparticles (Florence 1997). Third, their submicron size and their large specific surface area favor their absorption compared to larger carriers. Also, the particle surface can be modified by adsorption or chemical grafting of certain molecules such as poly(ethylene glycol) (PEG), poloxamers, and bioactive molecules (lectins).

Ahmad et al. (2018) prepared and evaluated PEGylated doxorubicin (DOX)-loaded poly-lactic-co-glycolic acid (PLGA) nanoparticles (NPs) for oral delivery. The nanoparticles had particle size, zeta potential, and drug content of 183.10 ± 7.41 nm, −13.10 ± 1.04 mV, and 42.69 ± 1.97 μg/mg, respectively. The pharmacokinetic study in rats showed 9.8 times improvement in relative bioavailability with PEGylated-DOX-PLGA-NPs as compared to plain drug suspension (Ahmad et al. 2018).

8.3.2 Liposomes

Liposomes were first described by English Hematologist Alec Bangham in 1961 and extensively used as delivery vehicles in pharmaceuticals. Liposomes are small vesicles of phospholipids and resemble the structure of biomembranes. Owing to their size and hydrophobic and hydrophilic character (besides biocompatibility), liposomes are advantageous systems for drug delivery (Zylberberg and Matosevic 2016).

Liposomes are used to enhance oral bioavailability of drugs and additional benefit provided by lipidic bilayer structure. They can adhere to biomembranes and form mixed micelle structures with bile salts which increase the solubility of lipophilic drugs. They have been successful in improving oral bioavailability by lymphatic uptake of various compounds such as peptide and proteins and hydrophilic and lipophilic drugs (Akbarzadeh et al. 2013).

Li et al. (2017) developed and evaluated itraconazole liposomes to enhance oral bioavailability using sodium deoxycholate by thin-film dispersion method. Liposome was spherical shaped with 118.1 ± 2.0 nm particle size and −21.5 ± 1.3 mV zeta potential. The bioavailability study in rats after oral administration showed that AUC was nearly 1.67-fold higher with liposome than that of marketed capsules (SPORANOX) (Li et al. 2017).

8.3.3 Lipid Nanoparticles

Lipid-based nanoparticles have gained much attention to improve the oral bioavailability of poorly water-soluble drugs. In comparison to other micro- or nanoparticulate-based carriers, lipid nanoparticles combine the advantages of both polymeric nanoparticles and emulsions, provide good tolerability and protection of active ingredient against chemical degradation, augment their oral bioavailability,

and are easy to scale up using high-pressure homogenization. These attributes make them an alternative carrier system to polymeric nanoparticles and liposomes (Puri et al. 2009). The different types of lipid nanoparticles are solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), lipid drug conjugates (LDCs), and Lipid-Polymer Hybrid Nanoparticles (LPNs). These different types of lipid nanoparticles offer a promising and versatile carrier system for oral delivery of lipophilic drugs.

8.3.3.1 Solid Lipid Nanoparticles (SLNs)

Professor R.H. Müller (Germany) and Professor M. Gasco (Italy) in the early 1990s have investigated the potential of solid lipid nanoparticles (SLNs), a first generation of lipid nanoparticles. The formulation composed of lipids and avoided the use of organic solvent during its fabrication, in contrast to existing organic nanoparticles (e.g., PLGA nanoparticles) (Muller et al. 2000).

SLNs are defined as "aqueous colloidal dispersions, the matrix of which composed of solid lipids, having the particle size range of 10–500 nm." Like nanoemulsion and liposomes, they are comprised of biocompatible excipients such as fatty acids and lipids (Harde et al. 2011). Because of its natural and biological origin, it has low toxicity than that of the polymeric particles (Sawant and Dodiya 2008). Like polymeric NPs, solid matrix of SLNs makes them stable and prevents the drug degradation in harsh environment of GIT. They have high entrapment efficiency and provide a controlled drug release up to several weeks. The production of SLNs can be scaled up using high-pressure homogenizer (HPH) (Blasi et al. 2007; Kaur et al. 2008). The small size range (around 10–200 nm) of SLNs can easily cross tight junctions of intestine cells and improves the BA of drugs. Also, site-specific delivery can be easily achieved by using specific ligand. All these characteristics of SLNs effectively make them an excellent carrier for oral drug delivery.

Dwivedi et al. (2014) prepared arteether (ART)-loaded SLN by high-pressure homogenization (HPH) technique. The particle size and EE were found to be 100 ± 11.2 nm and 69 ± 4.2%, respectively. The in vitro drug release profile of ART-SLNs was slow but time dependent which would help to protect the acid degradation of ART in stomach. The pharmacokinetics results indicated that absorption of ART-SLNs was significantly enhanced in comparison to ART in aqueous suspension and ART in ground nut oil (GNO) in rats (Dwivedi et al. 2014).

Patel et al. (2019a) developed and evaluated SLNs of asenapine maleate (AM) to improve its oral bioavailability (BA). They prepared AM-SLNs having particle size 114.3 ± 3.5 nm and entrapment efficiency of 84.10 ± 2.90%. In vitro release study of AM-SLNs showed 9.23 ± 2.72% in acidic medium and 92.09 ± 3.40% release of AM in phosphate buffer pH 6.8. Nontoxicity of the carriers and drug was proved by cell viability study across Caco-2 cells. The uptake of AM-SLNs across Caco-2 cell line was time and energy dependent exhibiting clathrin/caveolae-mediated endocytosis transport. Cellular uptake of coumarin-6-loaded SLNs was effectively increased as compared to the dye solution. The pharmacokinetic results in rats

showed 50.19-fold improvement in BA of AM after fabrication of SLNs (Patel et al. 2019a).

8.3.3.2 Nanostructured Lipid Carriers (NLCs)

To conquer the drawback of SLNs, i.e., drug expulsion during storage, use of an alternate strategy (mixture of lipids) which forms less crystalline arrangement is needed. Nanostructured lipid carrier (NLC) was developed in the late 1990s, as second generation of lipid nanoparticles. NLCs comprise of unstructured matrix containing blend of solid lipid and liquid lipid dispersed in an aqueous phase surfactant solution (Beloqui et al. 2016). The NLC matrix remains solid at room/body temperature but incorporation of liquid lipid forms more imperfections in the structure and can accommodate more drug as compared to SLNs. Owing to its solid matrix, it can strongly immobilize the drug and protect the coalescence of particles (Jaiswal et al. 2016).

NLC shows biphasic drug release pattern and initial burst drug release followed by a sustained drug release for longer time. The presence of liquid lipid at the outer layer NLC provides burst release of the drug. Unlike SLN, these oil-enriched outer layers hold more solubility for lipophilic drugs and hence, a higher amount of drug could be easily incorporated in NLC (Khan et al. 2015).

Shah et al. (2016) prepared raloxifene hydrochloride (RLX)-loaded NLCs by solvent diffusion method for improvement in oral bioavailability. The optimized batch showed 32.50 ± 5.12 nm average particle size, −12.8 ± 3.2 mV zeta potential, and smooth spherical particles. In vitro release study exhibited burst release followed by sustained release up to 36 h. In vivo pharmacokinetic study showed 3.75-fold enhancement in bioavailability than plain drug suspension (Shah et al. 2016).

8.3.3.3 Lipid Drug Conjugates (LDCs)

Incorporation of hydrophilic drugs in SLNs and NLCs is restricted because of presence of lipid core. Lipid drug conjugates (LDCs) have overcome this limitation. LDCs are defined as drug molecules that are covalently modified using lipids. LDC is used for the delivery of drugs with high drug loading capacity. The preparation method for LDCs involves (1) formation of an insoluble drug-lipid conjugate and (2) incorporation of LDC in an aqueous surfactant solution to produce nanoparticles using any technique, high-pressure homogenization (HPH). LDCs also provide several advantages including enhanced drug loading, improved oral bioavailability, enhanced tumor targeting, and reduced toxicity (Adhikari et al. 2017; Irby et al. 2017).

Agrawal et al. (2015) designed lipid drug conjugate (LDC) nanoparticles as autolymphotrophs for oral delivery of methotrexate (MTX) to improve its oral bioavailability. The optimized batch had particle size and entrapment efficiency of 152.1 nm and 82.8%, respectively. The lymphatic transport mechanism was the

predominant mechanism behind bioavailability enhancement of MTX as LDC-NPs significantly enhanced the retention time in intestinal lymphatics (Agrawal et al. 2015).

8.3.3.4 Lipid-Polymer Hybrid Nanoparticles (LPNs)

Lipid-polymer hybrid nanoparticles (LPNs), a novel carrier system, have been widely developed to overcome demerits of polymeric nanoparticles and liposomes. LPNs are polymeric nanoparticles covered by lipid layers which have integrated property of biocompatible lipids and integrity as that of polymeric nanoparticles.

The outer layer of lipid around the polymeric nanoparticles protects the release of water-soluble drugs and, hence, enhances their entrapment efficiency. Thus, presence of lipid in LPNs offers various advantages, such as (a) encapsulation of both lipophilic and hydrophilic drugs, (b) surface modification for specific targeting, and (c) fast drug release prevention of water soluble drugs (Cheow and Hadinoto 2011).

LPNs can entrap various molecules such as drugs, proteins, genes, and targeting ligands which can also be attached to the hybrid system. Different polymers such as polycaprolactone (PCL), polylactic-co-glycolic acid (PLGA), albumin, or dextran can be used for fabrication of LPNs owing to their nontoxicity, biocompatibility, and biodegradability. Usually lipids used are lecithin, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), or 1,2-dioleoy l-3-trimethylammonium-propane (DOTAP) (Mandal et al. 2016).

Suares and Prabhakar (2017) investigated potential of LPNs to improve oral bioavailability of rosuvastatin calcium (ROS). The optimized batch had particle size of 61.37 ± 3.95 nm, entrapment efficiency of 86.77 ± 1.27%, and zeta potential of −6.72 ± 3.25 mV. In vitro drug release study of ROS-loaded LPNs showed pH-independent sustained release of ROS from LPN in comparison to drug suspension. Intestinal permeability study showed prolonged drug release of ROS from LPN due to adhesion of polymer to mucus layer. In vivo absorption of ROS from LPNs resulted in 3.95-fold and 7.87 increase in AUC and mean residence time as compared to drug suspension. Furthermore, ROS-loaded LPNs reduced elevated lipid profile in rats (Suares and Prabhakar 2017).

8.3.4 Self-Emulsifying Drug Delivery System (SEDDS)

A self-emulsifying system composed of oil, surfactant and cosolvent that emulsifies in water under conditions of gentle agitation of GIT. SEDDS are isotropic mixtures of oils, surfactants, and/or cosolvents that rapidly produce o/w emulsions upon digestive motility of gastrointestinal tract. SEDDS is a broad term, generally producing emulsions with a droplet size in the range of 100–200 nm in the case of self-microemulsifying drug delivery systems (SMEDDS) and less than 100 nm in

the case of self-nanoemulsifying drug delivery systems (SNEDDS). Both SMEDDS and SNEDDS are isotropic mixtures of oil, hydrophilic surfactant, and cosolvents that rapidly form o/w microemulsions upon gentle agitation followed by dilution in an aqueous media that will be experienced in the GI tract (Dokania and Joshi 2015). They can be filled in gelatin (soft or hard) or hydroxy propyl methyl cellulose (HPMC) capsules made suitable for oral delivery.

Mundada and Sawant (2018) investigated potential of SMEDDS of dabigatran etexilate (DE) to improve its oral bioavailability. The optimized batch had globule size of 73.24 nm with 0.085 PDI. The in vitro and ex vivo drug release from DE-SMEDDS was found to be significantly higher in comparison to that from plain drug suspension. The DE-SMEDDS showed a deeper penetration in the Caco-2 cells using confocal laser scanning microscopy. Flow cytometric study showed greater uptake from the DE-SMEDDS as that of drug suspension. In vivo pharmacokinetic study in rats showed that $AUC_{0\rightarrow t}$ of DE-SMEDDS formulation was found 2.5 times higher and relative bioavailability was enhanced by 3.36-fold than that of drug suspension after oral administration. Moreover, anticoagulant activity of DE SMEDDS was higher than drug suspension, further indicating better bioavailability (Mundada and Sawant 2018).

Patel et al. (2019b) fabricated asenapine maleate (AM)-loaded SMEDDS (AM-SMEDDS) to increase its oral bioavailability. The AM-SMEDDS showed globule size and zeta potential of 21.1 ± 1.2 nm and − 19.3 ± 1.8 mV, respectively. In vitro drug release study showed 99.2 ± 3.3% of drug release in phosphate buffer pH 6.8 at the end of 8 h. Ex vivo drug release study showed only 15% of drug diffusion through stomach and ~85% drug was diffused through intestinal membrane. Uptake studies using confocal and flow cytometry study exhibited enhanced cellular uptake of coumarin-6-loaded SMEDDS across Caco-2 cells as that of coumarin-6 solution. The relative bioavailability of AM-SMEDDS was found to be 23.53 times greater than AM suspension. Intestinal lymphatic transport study using cycloheximide (CHX) showed that the AUC_{total} of AM-SMEDDS reduced about 35.67% compared with that without the treatment of CHX indicating involvement of lymphatic system in intestinal absorption of AM-loaded SMEDDS (Patel et al. 2019c).

8.3.5 Polymeric Micelles (PMs)

PMs are self-assembled core-shell nanostructures formed in an aqueous solution consisting of amphiphilic block copolymers. PMs enhance drug absorption via different mechanisms which include (1) protecting drug degradation from harsh environment of the GI tract, (2) controlled drug release at target site, (3) prolongation of the residence time in the gut by mucoadhesion, and (4) inhibitory effect of efflux pumps (Xu et al. 2013).

Fares et al. (2018) prepared, optimized, and evaluated lacidipine-loaded polymeric micelles (LCDP-PMs) using thin-film hydration technique to enhance solubility-limited oral bioavailability. The optimized batch had particle size of

21.08 nm and entrapment efficiency of 99.23%. The saturation solubility was 450 times with LCDP-PMs as that of pure LCDP and dissolution rate was also significantly enhanced. Bioavailability study in rabbits revealed a 6.85-fold increase in LCDP bioavailability with LCDP-PMs compared to LCDP oral suspension (Fares et al. 2018).

8.4 Applications

8.4.1 Cancer

Cancer is the uncontrollable division of abnormal cells which may spread to nearby healthy tissues and organs. Tumor cells show overexpression of tumor-specific antigens (TSA) whereas an endogenous antigen, tumor-associated antigens (TAA), is present on both tumor and on normal cells. Recently, nanotechnology has been widely explored for anticancer drug delivery for improving the efficacy of chemotherapy and reducing side effects to healthy cells (Ahmed et al. 2012).

Pooja et al. (2016) prepared wheat germ agglutinin (WGA)-conjugated, solid lipid nanoparticles to improve the oral delivery of paclitaxel (PTX). The in vitro drug release study showed sustained drug release pattern up to 96 h. They reported that anticancer activity of PTX-loaded SLNs was effectively enhanced against A549 lung cancer cells. Intracellular uptake study showed higher fluorescence with conjugated SLNs than unconjugated SLNs. Moreover, the biodistribution studies in rats revealed that the oral bioavailability and lung targetability of PTX by conjugated SLNs was significantly improved due to bioadhesive property of the nanocarrier system and presence of targeting ligand WGA (Pooja et al. 2016).

Joshi et al. (2014) designed and evaluated gemcitabine HCl-loaded PLGA nanoparticles for enhanced oral bioavailability. The optimized batch had particle size and EE of 166.4 ± 2.42 nm and 56.48 ± 3.63%, respectively. Uptake study using confocal microscopy showed enhanced absorption and uptake of NPs across Caco-2 cell line. Transport study demonstrated 6.37-fold enhancement of permeability of NPs. MTT assay demonstrated significant cytotoxicity of NPs on K562 leukemia cell lines. In vivo pharmacokinetic studies in rats showed 21.47-fold bioavailability enhancement from NPs (Joshi et al. 2014).

8.4.2 Neurodegenerative Diseases

Treatment of neurodegenerative disease is difficult due to unfavorable pharmacokinetics and pharmacodynamics of drugs. The several reasons which might be responsible for treatment failure include physicochemical property of drugs (hydrophobicity), poor absorption by biological membranes, unfavorable

pharmacokinetic parameters (metabolism), instability of drugs (oxidation, hydrolysis, or photolysis), and toxicity to tissues (hepatotoxicity, neurotoxicity, or kidney toxicity). The use of nano-based formulations aids in their pharmacokinetic and pharmacodynamic parameters, minimizes their toxicity, and provides controlled drug release (Fonseca-Santos et al. 2015; Cacciatore et al. 2016; Joseph et al. 2017).

Joseph et al. (2017) developed and evaluated olanzapine-loaded solid lipid nanoparticles for the treatment of schizophrenia. SLNs were developed using glyceryl monostearate by emulsification-ultrasonication technique. The solid lipid nanoparticles had PS of 151.29 ± 3.36 nm with EE of 74.51 ± 1.75%. In vitro drug release study showed extended drug release up to 48 h. In vivo studies have demonstrated an improved therapeutic efficacy than pure olanzapine (Joseph et al. 2017).

Misra et al. (2016) fabricated solid lipid nanoparticles of galantamine hydrobromide to improve its pharmacokinetic profile in the treatment of Alzheimer's disease (AD). The developed SLNs offered mean particle size lower than 100 nm with drug entrapment of 83.42 ± 0.63%. In vitro drug release was more than 90% for a period of 24 h in controlled manner. In vivo pharmacodynamic study demonstrated significant memory restoration in cognitive deficit rats in comparison with pure drug. The pharmacokinetic study of SLNs offered twofold enhanced bioavailability to that of plain drug (Misra et al. 2016).

Patel et al. (2019c) prepared and evaluated the efficacy of solid lipid nanoparticles (SLNs) to enhance the absorption and bioavailability of lurasidone hydrochloride (LH) following oral administration. The LH-SLNs showed PS of 139.8 ± 5.5 nm and EE of 79.10 ± 2.50%. The in vitro release from LH-SLNs followed Higuchi model. The ex vivo permeability study demonstrated enhanced drug permeation from LH-SLNs (>90%) through rat intestine as compared to LH suspension. The SLNs were found to be taken up by energy-dependent endocytic mechanism which was mediated by clathrin/caveolae-mediated endocytosis across Caco-2 cell line. The pharmacokinetic results showed that oral bioavailability of LH was improved over 5.16-fold after incorporation into SLNs as compared to LH suspension. The pharmacodynamic study proved the antipsychotic potential of LH-SLNs in the treatment of schizophrenia. Authors proved that oral administration of LH-SLNs in rats improved the bioavailability of LH via lymphatic uptake along with improved therapeutic effect in MK-801-induced schizophrenia model in rats (Patel et al. 2019b).

8.4.3 HIV/AIDS

The advent of a class of protease inhibitors and the introduction of triple-drug therapy in the mid-1990s have revolutionized HIV/AIDS treatment. Nowadays, the highly active antiretroviral therapy (HAART) has resulted in tremendous success in improving the therapeutic efficacy and patient lives. But HAART regimens have serious side effects and HAART therapy has to be taken for a lifetime at daily dose of one or more pills (Mamo et al. 2010).

Nanotechnology-based drug delivery systems for systemic delivery of antiretroviral drugs provide controlled drug release which can enhance their half-life and keep them in circulation for longer periods of time. The targeted delivery of antiretroviral drugs by their surface modifications to CD4+ T cells and macrophages enhances their therapeutic efficacy (Boyapalle et al. 2012).

Venkatesh et al. (2015) developed and evaluated nelfinavir-loaded PLGA nanoparticles using nanoprecipitation technique to enhance its oral bioavailability and therapeutic efficacy. The developed NPs showed PS, zeta potential, and EE of 185 ± 0.83 nm, 28.7 ± 0.09 mV, and 72 ± 0.47%, respectively. In vivo bioavailability study in rabbits exhibited 4.94-fold improvement with nanoparticles as that of pure drug suspension (Venkatesh et al. 2015).

Gaur et al. (2014) investigated potential of SLNs to increase bioavailability of efavirenz, a non-nucleoside inhibitor drug. The efavirenz-loaded SLNs showed average particle size of 124.5 ± 3.2 nm with a PDI value of 0.234% and 86% drug entrapment. In vitro drug release study showed controlled drug release up to 24 h. In vivo pharmacokinetic study in rats exhibited 5.32-fold increase in peak plasma concentration (ŭ36;max) and 10.98-fold increase in AUC in comparison to efavirenz suspension (Gaur et al. 2014).

8.4.4 Diabetes

Nanotechnology-based medicine has enabled more robust insulin delivery that can detect fluctuations in blood glucose levels (BGLs) and automatically modulate the rate of insulin release to maintain normoglycemia (He et al. 2013).

Damge et al. (2007) developed polymeric nanoparticles of insulin using a blend of a (poly(−ε-caprolactone)) and a Eudragit® RS. Insulin-loaded nanoparticles showed a mean diameter of 358 ± 12 nm with high encapsulation efficiency of 96.3 ± 0.42%. Insulin nanoparticles decreased the fasted glycemia in diabetic rats in a dose-dependent manner and also improved the glycemic response for prolonged time. It was observed that FITC-Insulin-loaded nanoparticles remain adhered to the intestinal mucosa and labeled insulin was taken up by the Peyer's patches (Damge et al. 2007).

Shi et al. (2016) prepared NLCs of baicalin to improve its antidiabetic action. The NLC showed PS of 92 ± 3.1 nm, PI of 0.13 ± 0.02, and zeta potential of −31.35 ± 3.08 mV. In vivo antidiabetic activity in rats showed marked reduction in FBG (fasting blood glucose) level and was found to be effective in reducing diabetes (Shi et al. 2016).

8.4.5 Hypertension

Hypertension is a serious cardiovascular event which refers to rise in the arterial blood pressure. Conventional dosage forms of antihypertensive drugs are available but majority of the antihypertensives are poorly water soluble and therefore exhibit low bioavailability. These drugs are substrate of P-gp and undergo extensive first

pass metabolism. Also, they have short half-life and high dosing frequency. These drawbacks can effectively be overcome by utilizing benefits provided by nanoparticles which include controlled drug release, reduction in first pass metabolism, and p-gp efflux (Alam et al. 2017).

Dudhipala and Veerabrahma (2016) prepared and evaluated candesartan cilexetil-loaded solid lipid nanoparticles (CC-SLNs) to overcome poor aqueous solubility and low oral bioavailability. The CC-SLNs had PS of 180–220 nm and EE of 91–96%. DSC and XRD analysis indicated that the drug was in amorphous form in SLNs. The pharmacokinetic study in rats showed that bioavailability of CC was improved over 2.75-fold after fabricating SLNs. The pharmacodynamic study in hypertensive rats showed a decrease in systolic blood pressure for 48 h and 2 h with CC-SLNs and drug suspension, respectively (Dudhipala and Veerabrahma 2016).

Ranpise et al. (2014) developed lercanidipine hydrochloride-loaded nanostructured lipid carriers to enhance its oral bioavailability. The NLC exhibited mean size of 214.97 nm and a zeta potential of −31.6 ± 1.5 mV. The ex vivo drug release study showed that the drug release was enhanced from 10% to 60.54% at blood pH in 24 h. The in vivo pharmacodynamic study in rats showed that controlled drug release from NLCs as compared to plain drug suspension (Ranpise et al. 2014).

8.5 Conclusion

Nanotechnology-based formulation is an emerging tool to mitigate the drawbacks associated with oral delivery of lipophilic drugs. Various formulations based on nanotechnology such as polymeric nanoparticles, liposomes, SLNs, NLCs, LDCs and LPNs can effectively aid the oral absorption and permeation and thereby improve the therapeutic efficacy of poorly water-soluble drugs. All these formulations also have proved their potential in BA enhancement by reduction in particle size, increasing water solubility, surface modification, enhancing permeability, and reducing first pass metabolism. Thus, nanotechnology-based formulations hold a strong potential in the development of effective oral drug delivery systems for bioavailability enhancement in the treatment of various diseases.

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Chapter 9
Nanotechnology in Early Detection and Treatment of Amyloidosis

Maryam Yousaf, Muhammad Ahmad, Ijaz Ahmad Bhatti, and Nasir Mahmood

Abstract Quick detection and effective modulation of amyloidosis is a prerequisite in current world. In this context, nanotechnology is playing a major role by designing appropriate nanoparticle (NP)-based theranostics that will help to effectively surpass the hurdles faced by old diagnostic and therapeutic strategy, i.e., low detection limit to diagnose monomers and oligomer (main culprit of amyloidosis) of amyloid peptide and inability of drug to cope up with body environment, short life time, and reduced capability to cross blood-brain barrier (BBB). Most prominent amyloidosis-related diseases, i.e., type 2 diabetes (T2D) and Alzheimer disease (AD), are discussed in this chapter. Effects of both diseases are almost irreversible and difficult to diagnose at early stages, making its timely treatment difficult, which will be highlighted in this chapter. Nanotechnology plays a vital role in this context; development of both in vitro and in vivo nanodiagnostic and treatment methods is enabling early diagnosis and up to 10 fg/mL detection limit of AD and T2D biomarkers. Although the role of nanotechnology as nanotheranostics is quite obvious but still some challenges persists that needs to be addressed; e.g., NPs instead of inhibiting amyloidosis process may act as a seed/catalyst and speed up amyloidosis; all these will be discussed in this chapter. Moreover, effect of surface chemistry, size and hydrophobicity of NPs over their interaction with amyloid protein and control of amyloidosis will also be explained in this chapter. Moreover, limited literature is available for using NPs as in vivo nanotheranostics; therefore there is a need to

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_9

conduct more studies about the use of NPs as in vivo theranostics considering its cytotoxicity and correlation of in vivo and in vitro studies. Thus, in the near future, clinical trials should be extended to humans to evaluate the evaluation efficacy and safety of NPs as nanotheranostics that will help to design a promising and cost-effective strategy.

Keywords Amyloidosis · Alzheimer disease · Amyloid aggregation · Nanotheranostics · Nanodiagnostics · Type 2 diabetes

9.1 Introduction

Amyloidosis is a vast group of diseases characterized by insoluble amyloid fibrils, which forms extracellular deposits in body organs and tissues. These fibrils are formed by pathological misfolding of specific precursor proteins from their soluble tertiary structure into insoluble amyloid fibrils. Generally, the term "amyloid" is referred to any type of peptide fibrils containing characteristic cross-β-sheet structure, independent of their extracellular or intracellular location and disease association (John et al. 2018; Pansieri et al. 2018). General structure of fibrils contains two adjacent β-sheets that are 8–11 Å apart with 4.7 Å interstrand distance within one sheet and a common secondary structure motif. Amyloid fibrils further undergo hydrophobic and hydrogen-bonding interaction to form network of aggregates named as "amyloid plaques." Different amyloid precursor proteins abnormally folded into amyloid aggregates responsible for different types of amyloidosis (Alvarez et al. 2013). Despite involvement of different diseases in amyloidosis, amyloid fibrils formed in these diseases share same structural (hydrogen-bonded stacks of β-sheets) and morphological (containing 2–6 protofilament of 2–5 nm wide that form unbranched fibrils) features (Nelson et al. 2005; Adamcik and Mezzenga 2011).

9.2 Types of Amyloidosis and Their Impacts

Initially mature insoluble amyloid fibrils were considered main culprit for the amyloidosis but recent studies have shown that amyloid aggregation (AA) into fibrils occurs through a number of complicated steps, which involved various intermediate product formations, i.e., oligomeric species and supramolecular assemblies that possess significant cytotoxicity (Bhattacharya et al. 2013). These intermediate products either lead to the AA into mature fibrils or follow an off-pathway, product pathway possessing varying degree of cytotoxicity (Fig. 9.1).

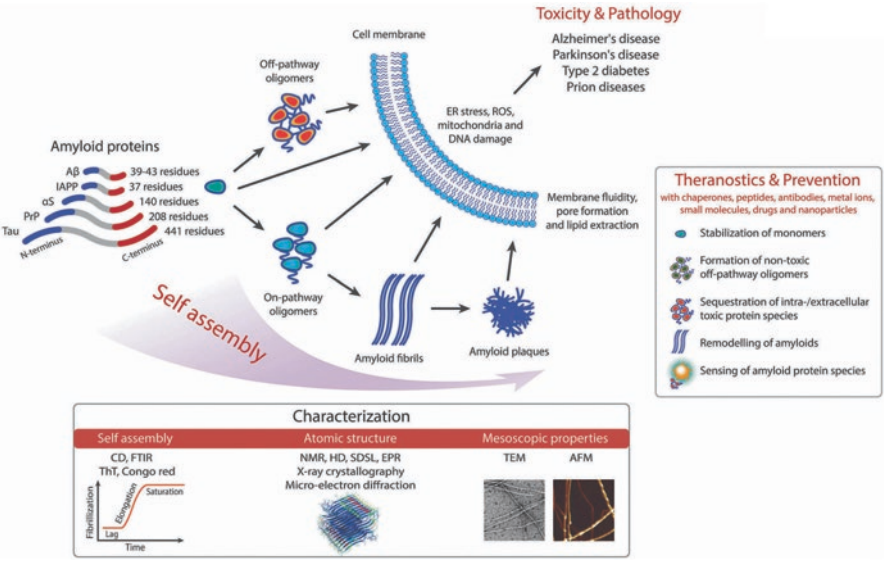

Fig. 9.1 Amyloid proteins self-assembly pathway highlighting self-assembly impacts, theranostics, and methods of detection of amyloid fibrils. Reproduced with permission (Ke et al. 2017), copyrights reserved to The Royal Society of Chemistry 2017

9.2.1 Types of Amyloidosis

Cytotoxicity of these oligomers and fibrils results in amyloidosis that is classified into different types depending upon biochemistry of amyloid protein involved and their clinical signs. Amyloidosis might be primary (occurring on its own) or secondary (developed due to other medical conditions), localized, and multisystemic, whereas ratio of developing primary and secondary amyloidosis is 1:9. To date, 36 proteins in the human body are known to be amyloidogenic and are responsible for systemic, intermittent, or genetic disorders without any functional and structural connection (Sipe et al. 2016). Moreover, all fibrils show affinity for Congo red staining and show apple-green birefringence after being stained by Congo red that can be observed under a polarized light microscope. Different amyloid proteins, i.e., β_2-microglobulin, islet amyloid polypeptide, and calcitonin, are responsible for metabolic diseases (hereditary visceral, T2D, and carcinoma of the thyroid), whereas amyloid beta (Aβ), alpha-synuclein, and prion protein are responsible for neurodegenerative diseases (Parkinson's disease, AD, and Creutzfeldt-Jakob disease). There is another group of amyloid proteins which spontaneously form aggregates such as ApoAI, ApoAII, and ApoCII (apolipoprotein fragments) and are associated with atherosclerosis.

9.2.1.1 Alzheimer Disease and Type 2 Diabetes

The AD and T2D are the most commonly studied disease among amyloid diseases because of their global diversity (47 and 382 million people are suffering from AD and T2D, respectively), clinical impact, and metabolic correlation with each other (Pansieri et al. 2018). In 1907 a German neuropathologist Alois Alzheimer reported the world's first AD disease patient (Stelzmann et al. 1995). AD is a neurodegenerative disease characterized by neurofibrillary tangles (NFT) (aggregates of tau proteins into dead neurons) in nerve cells and senile plaques (SP) of amyloid-beta (Aβ) protein. Hence, aggregation of Aβ fibrils was considered as the hallmark of AD that leads to the synaptic function impairment. In addition, free radicals, oxidative stress, and disruption in functions of the autophagy system are also responsible for pathogenesis of AD (Nazem and Mansoori 2011; Kao et al. 2015). People suffering from AD exhibit several symptoms, e.g., memory impairment, personality changes, confusion, and impaired judgment. It is the most common form of senile dementia and has become an important medical and social issue (Stelzmann et al. 1995). Diabetes is a metabolic disorder that occurred either due to insulin deficiency (type 1 diabetes) whereas another type of diabetes (T2D/adult-onset diabetes) is developed with hyperinsulinemia/insulin resistance that ultimately results in high blood glucose level; around 90% of people are suffering from T2D (Marchetti et al. 2008; Radovan et al. 2009). A T2D is developed either due to genetic factor or obesity (acquired) factor; another contributing factor in development and progression of T2D is fibrillar deposits of human islet amyloid polypeptide (hIAPP) in pancreatic β-cell. That will lead to death and disruption in functioning of pancreatic β-cells (enhanced/abnormal insulin secretion) ultimately leading to progression of T2D (Kahn 2003; Haataja et al. 2008; Ahmad et al. 2011).

9.2.1.2 Correlation Between Alzheimer Disease and Type 2 Diabetes

Currently, T2D and AD are serious health issues, although both diseases vary in symptoms (AD is neurodegenerative; T2D is metabolic disease) but share similar causes of development (living style mismanagement, similar pathological conditions such as insulin sensitivity, deficiency, and/or metabolism) and mechanism. Clinical evidence and studies in different countries like the USA, Taiwan, Japan, China, and Finland confirmed that people suffering from T2D are at a risk of developing AD. Therefore, by controlling the development of T2D cognitive impairment/pathology of AD can be delayed or prevented. AD and T2D are linked to each other through glucose and insulin metabolism load to the level of Aβ. Increase glucose level leads to enhanced/increase Aβ levels in people suffering from AD (Song et al. 2017). Main cause of AD development in diabetic people is aggregation of hIAPP, where clinical evidence clearly shows that along with mature IAPP fibrils dense senile plaques of Aβ have also been observed in the brain of person suffering from

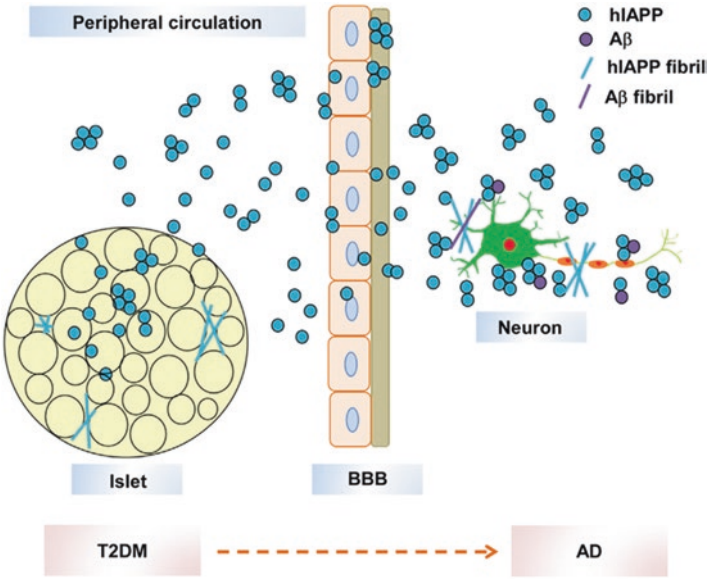

Fig. 9.2 Mechanistic overview of development of AD because of IAPP aggregation. Reproduced with permission (Zhang and Song 2017), Copyrights reserved to Elsevier Inc. 2017

T2D (Fig. 9.2). Mechanistically, IAPP fibrils and oligomers through blood circulation approached BBB and enter into the brain by destroying integrity of BBB that ultimately results in the brain impairment (Jackson et al. 2013; Srodulski et al. 2014; Oskarsson et al. 2015). Hence, hIAPP aggregation should be stopped, to halt the development of other neurodegenerative diseases, mainly AD.

9.2.1.3 Mechanism of Amyloid Aggregation in Alzheimer Disease and Type 2 Diabetes

Due to rapid spread of AD and T2D it is necessary to design a therapeutic strategy that helps to control the development of these diseases. A valuable amyloidosis treatment strategy can be designed by understanding the structure and aggregation mechanism of amyloid protein. Amyloid fibril formation can be effectively monitored/observed by microscopy and spectroscopy techniques (Hong et al. 2012; Yousaf et al. 2018). Generally, AA starts with the peptide's monomers and then follows different AA nucleation mechanisms, i.e., nucleated polymerization (NP)/classical nucleation theory and nucleated conformational conversion (NCC) (Serio et al. 2000; Gillam and MacPhee 2013). AA by NP followed sigmoidal growth curve and starts with "lag phase" (primary nucleation phase), where soluble

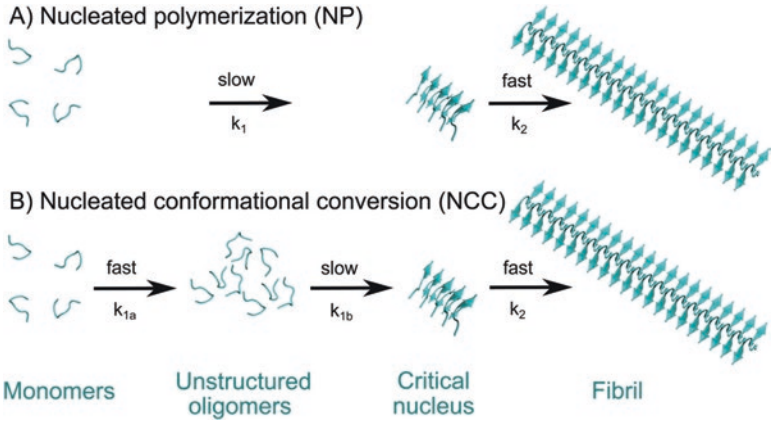

Fig. 9.3 Different models describing the AA mechanism. (**a**) NP: highly ordered β-sheet-containing oligomers of critical size are formed during rate-determining step (slow step); these oligomers rapidly assembled into mature fibrils (fast step). (**b**) NCC: monomers aggregate to form unstructured oligomers (fast step); these unstructured oligomers during rate-determining step aggregate to form critical β-sheet-rich oligomers (nuclei) (slow step) that quickly grow to form mature fibrils. Reproduced with permission (John et al. 2018), Copyrights reserved to Royal Society of Chemistry, 2018

monomers interact to form small soluble aggregates, i.e., nucleation seeds (oligomers). Monomers continue interacting until critical nucleus size (n_c) of oligomers is reached that starts exponential growth phase (fibrils growth) where length of resulted fibrils starts increasing by addition of monomers (Fig. 9.3). Moreover, nucleation phase of AA can be catalyzed by seeding peptide solution with either mature fibrils or nuclei (n_c), called secondary nucleation. In this case surface of existing fibrils/nuclei act as template, while catalyst catalyzes the formation of new soluble aggregates, thus speeding up AA process. Furthermore, AA is also affected by fragmentation process; during this process the number of fibrils is increased by breakdown of already present fibrils. All these microscopic events contribute differentially to the aggregation process and highly dependent upon amyloid protein aggregates (Knowles et al. 2009; Giorgetti et al. 2018; Linse 2019). On the other hand, if AA occurred by NCC, instead of formation of n_c of oligomers as intermediate, unstructured and loosely bound oligomers are formed in concentration-dependent manner. These oligomers in later stage restructured into well-ordered β-sheet-rich nuclei oligomers (protofibrils) that subsequently assembled into mature fibrils. Conclusively, NP model describes assembly of monomers into highly ordered oligomers of critical size whereas according to NCC model unstructured oligomers rearranged into highly ordered mature fibrils (Fig. 9.3) (Kelly 2000; Serio et al. 2000).

Aβ is one of the pathological features of AD; its aggregation follows both NP and NCC mechanism. Whereas hIAPP aggregation follows NP pathway, under pathological conditions, Aβ/hIAPP monomers spontaneously transform into a β-sheet-rich oligomeric structure (nucleus) that is accumulated in the brain and then arranged in an orderly manner to form fibrils visible under the microscope (Jerrett and Lansbury Jr 1993; Lee et al. 2011; Gillam and MacPhee 2013). Formation of oligomers is a rate-limiting step in the process of misfolding and aggregation of Aβ. The nucleus formation rate is very slow, but once the formation of protein is concentrated, it becomes rapid. Because the nucleus will self-assemble, which results in the misfolding and superimposing of nearby normal protein, ultimately the sampled oligomer will be longitudinally elongated along the fiber axis. In addition, the layers of sheet stacked perpendicularly to the fiber axis, eventually forming a high molecular weight unbranched insoluble fiber. According to NCC mechanism for Aβ aggregation, Aβ peptide rapidly forms unstructured oligomers that are slowly converted into the mature fibrils through NCC process (Lee et al. 2011).

9.2.1.4 Impacts of Alzheimer Disease and Type 2 Diabetes

Physiologically AD is identified by synaptic loss, cognitive decline, Aβ plaques, and NFT that ultimately leads to the death of neurons and loss in brain cell mass. Mainly, Aβ aggregates are accumulated at synapses, thus disrupting the neuronal pathway. Moreover, during the production of Aβ aggregates different pathways are followed that ultimately reduce the drug action given for AD treatment. In addition to this, Aβ oligomers are more toxic; these oligomers alter the protein functions mainly involved in fusion and fission of mitochondria that ultimately lead to neuronal death. In general, Aβ peptide disrupts mitochondrial functioning by destroying its respiratory pathway, increases oxidative stress, alters calcium handling power, and starts cell apoptotic signals. Moreover, intracellularly, accumulation of Aβ peptide affects the lysosomal efficiency to remove unfolded proteins, dead cells, and organelles (Fig. 9.4a).

Toxicity of IAPP aggregation is also associated with T2D, no toxicity is associated with smaller-sized oligomers, but this is not a denial from their involvement. Moreover, oligomeric species >100 nm do not form amyloid aggregates, whereas smaller monomeric species assembled over the membrane surface leading to formation of amyloid fibrils that produces pores over membrane leading to membrane disruption and death of β-cells leading to progression of diabetes (Fig. 9.4b) (Soong et al. 2009).

Understanding of AA mechanism leads to the development of several therapeutic strategies, basically stabilization of amyloid peptides monomers is the key element to control AA. Aggregation of hIAPP and Aβ leads to the development of two most dominant forms of amyloidosis (AD and T2D). Inhibition of hIAPP is a marvelous therapeutic strategy to stop β-cell death and halt the progression of T2D.

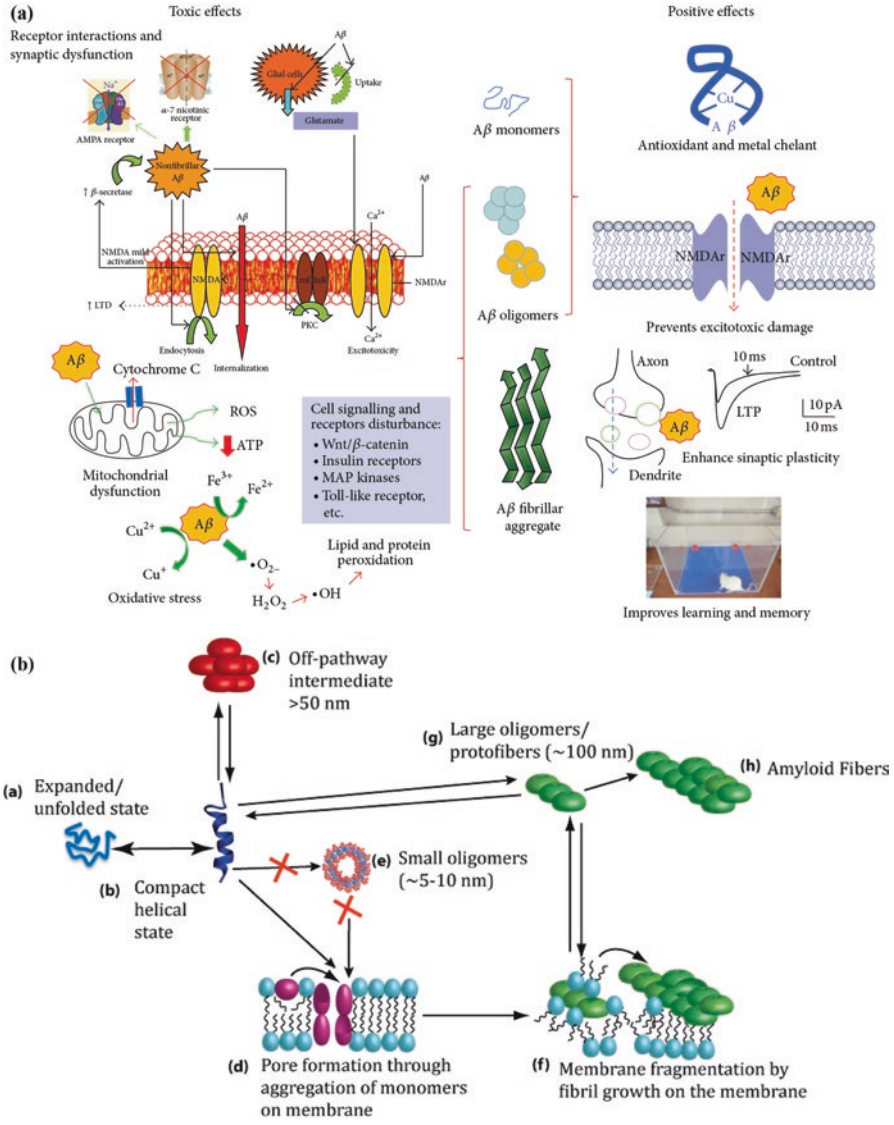

Fig. 9.4 Toxic effects of aggregation of Aβ. (**a**) Reproduced with permission (Carrillo-Mora P et al. 2014) Copyrights reserved to Hindawi Publishing Corporation 2014 and IAPP (**b**) Reproduced with permission (Soong et al. 2009), Copyrights reserved to American Chemical Society 2009

A number of chemicals, synthetic peptides, and compounds have been used as anti-amyloidogenic agents (Scrocchi et al. 2002; Mishra et al. 2008; Jeong et al. 2010; Cheng et al. 2012). However, high toxicity, surface passivation, and poor water dispersibility are the big hurdles in their application. Therefore, to use these materials as anti-amyloidogenic material, their water dispersibility and biocompatibility

should be enhanced. In this context, emerging field of nanotechnology is drawing great attention of researchers to use nanomaterials as anti-amyloidogenic agents due to their high biocompatibility, rich surface chemistry, and smaller size that allows nanomaterials to easily enter into cells and organelles allowing them to easily control in vivo AA. Limited literature is available regarding utilization of nanomaterials as an anti-amyloidogenic agent because of toxicity issues that hinder their application as an anti-amyloidogenic agent. Nanomaterials could either enhance AA or stop it by acting as an inhibitor (Chan et al. 2012; Nedumpully-Govindan et al. 2016).

9.3 Role of Nanotechnology in Detection and Treatment of Alzheimer Disease and Type 2 Diabetes

To reduce the number of pathogenic diseases developed by amyloid fibril formation, timely diagnosis and modulation of AA process is a hotspot of today's research. Up till now, different microscopic and spectroscopic methods are available to detect AA at early stages and monitor its kinetics.

9.3.1 In Vitro and In Vivo Diagnosis of Alzheimer Disease and Type 2 Diabetes

The difficulties of early diagnosis and nonavailability of efficient treatment methods lead to uncontrolled progression of AD and T2D. Available drugs for AD treatment failed due to their inability to crosss BBB and approach to amyloid plaques that ultimately leads to AD progression and brain impairment. AD is either detected by determining the concentration of Aβ in cerebral spinal fluid (CSF)/plasma or by determining whole tau protein. This approach can lead to false-positive results or is sometimes unconvincing because of the presence of other biological markers. Timely diagnosis of these diseases is essential to halt or prevent their progression. In this context, emerging field of nanotechnology is very helpful because it allows both in vitro and in vivo diagnosis of Aβ and hIAPP (markers for AD and T2D) aggregates or its oligomeric forms. The use of nanomaterials for diagnosis is based on their physicochemical properties, i.e., electrical, magnetic, optical, and biological response (Ahmad et al. 2017).

The aggregations of hIAPP and Aβ and their monomeric/oligomeric forms particularly of Aβ can be monitored both in vitro and in vivo by designing a simulation experiment or by taking a real animal model, respectively. For in vitro sensitive detection of Aβ plaques/fibrils in CSF, Georganopoulou and co-workers devised a bio-barcode technique (DNA-AuNP complexes). DNA-AuNP conjugates enable the detection of ultralow concentration of protein biomarkers. The DNA-AuNP conjugate only binds with the specific antibodies of biomarkers; these signals are

further amplified by the polymerase chain reaction (PCR) technique to enhance the signal for sensitive detection. This technique can successfully detect Aβ-derived diffusible ligands (ADDL) in CSF with sensitivity range of 10–18 M/L (Georganopoulou et al. 2005). Later on, a molecular detection system was designed that depends on scanning tunneling microscope (STM) electrical signals and two-photon Rayleigh spectroscopy for the detection of Aβ and tau protein at concentrations as low as 10 fg/mL and 1 pg/mL utilizing AuNPs (Kang et al. 2009; Neely et al. 2009). Localized surface plasmon resonance (LSPR)-based nanosensor utilizing metallic NPs (gold and silver NPs) has been developed by Moghimi (2011) for detection of ADDL to diagnose AD. A microfluidic biosensor technology and a sensitive electrochemical technology based on AuNPs were developed by Liu et al. (2013). According to Liu and co-workers developed biosensor ($A\beta_{1\text{-}16}$-heme-modified AuNPs) can detect $A\beta_{42}$ up to 100–300 μM using cyclic voltammetry upon adsorption of $A\beta_{42}$ on AuNPs. Further $A\beta_{1\text{-}16}$-heme-modified AuNPs can detect Aβ with the detection limit of 10 pM.

Similarly, literature study reported colorimetric method for hIAPP aggregation monitoring using resveratrol-modified AuNPs (Res-AuNPs). Res-AuNPs upon binding with hIAPP fibrils show reduction in absorbance at their 537 nm (λ_{max}). This reduction in absorbance is attributed to the decrease in concentration of free Res-AuNPs in supernatants (Zhang et al. 2016). Moreover, Rajasekhar and co-workers reported a nontoxic in vitro coumarin-quinoline (CQ) conjugate near-infrared (NIR) fluorescent probe for selective detection of amyloid plaques, which showed a preferable binding with amyloid plaques over α-synuclein and IAPP. A CQ probe can detect Aβ aggregates up to concentration of 86 nM that is ~10-fold higher than normal reported detection probe (thioflavin T) (Fig. 9.5) (Rajasekhar et al. 2017). Further advancement in amyloid aggregates detection is made by Liu et al. (2018), they have recently designed an interface containing 3D nanostructured immunoparticle for Aβ detection, which has shown promising response to aggregated fibrils.

Optical imaging (OI) and magnetic resonance imaging (MRI) are also used as in vivo nanodiagnostic tool to diagnose AD. Iron oxide-based magnetic NPs (IONPs/MNPs) are strong MRI probe and used as a contrast enhancer in MRI. The IONPs cross BBB upon intravenous infusion in AD mice and enhance MRI contrast. IONPs and ultrasmall SIONPs both are used as a contrast enhancer in MRI. The IONPs are not only used in their native form but also modified with antibodies to enhance their binding with the amyloid plaques and as MRI contrast enhancement agent. The conjugation of IONPs with antibodies enhances their ability to detect amyloid aggregates by twofold compared to the control group. This technique is not fully developed and adopted for an early detection mechanism because MRI detects amyloid plaques that are developed at advance stage of amyloid disease. However, this technique can be adopted for the detection of Aβ monomers/oligomers by decorating MRI contrast agents with specific antibodies that have ability to bind with oligomers/monomers. The oligomer detection by MRI technology makes it a superior technology over positron emission topography (Sillerud et al. 2013). Apart from MRI, another approach being used for the in vivo Aβ imaging/detection based on near-infrared (NIR) fluorescent dyes is optical imaging (OI) of molecular

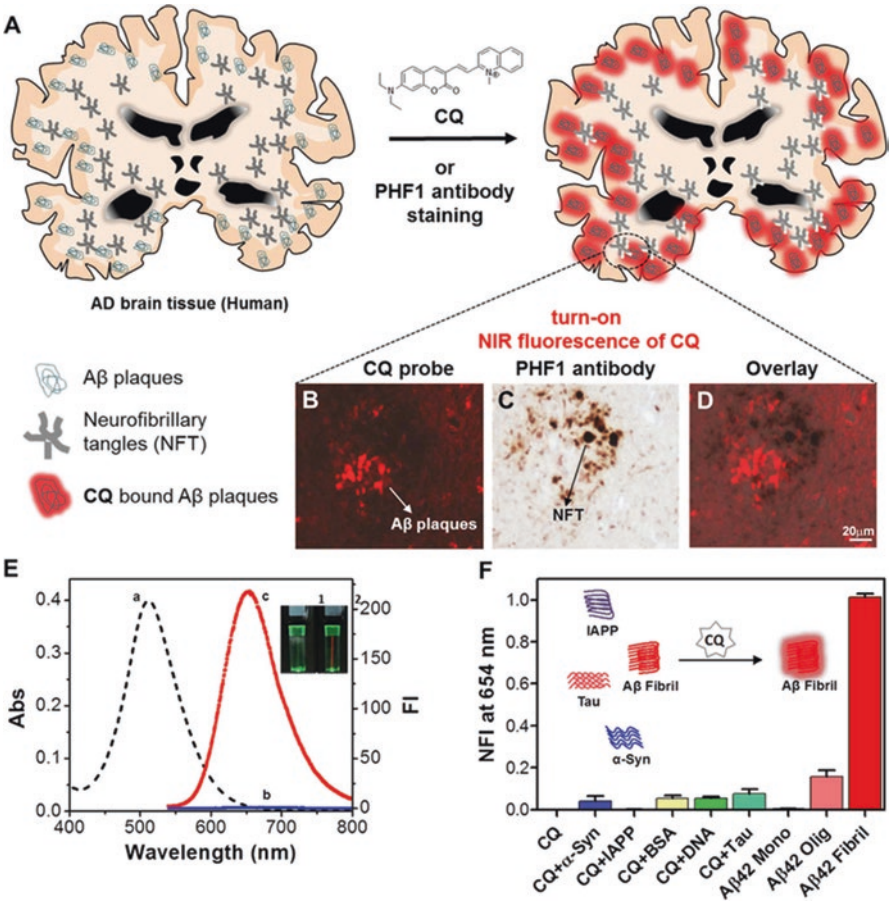

Fig. 9.5 Coumarin-quinoline (CQ) conjugate fluorescent probe (red and near and IR) for the detection of AD (A) CQ can in vitro selectively stain Aβ plaques. (B–D) PHF1 antibody stained neuritic component amyloid plaque is only stained with (C). The merged image shows there is no colocalization of PHF1 and CQ compound (D). (E) CQ fluorescence absorption (a) and emission (λ_{em} = 654 nm) at λ_{ex} = 521 nm spectra with (c) and without (b) $A\beta_{42}$ fibrillar aggregates. (F) CQ normalized fluorescence intensity upon interaction with different amyloid proteins. Reproduced with permission (Rajasekhar et al. 2017), Copyrights reserved to Elsevier Inc. 2017

biomarkers in various ailments including AD mice. Basic requisites for AD diagnosis involved the ability of probe to cross blood-brain permeability and specificity for the AD biomarkers. Among different NIR probes, few are used more commonly as NIAD-4, ThT-encapsulated polystyrene-block-poly(n-butyl cyanoacrylate) (PS-b-PnBCA) NPs, semiconductor quantum dots (QDs), transferrin-conjugated QDs, and polyethylene-coated QDs are being used for the detection of AD (Nazem and Mansoori 2011). Moreover, few more specified NIR imaging probes, e.g., oxazine derivative (Hintersteiner et al. 2005), NIAD-11, NIAD-4, and NIAD-16 dyes (Raymond et al. 2008), have also been developed for monitoring aggregation of

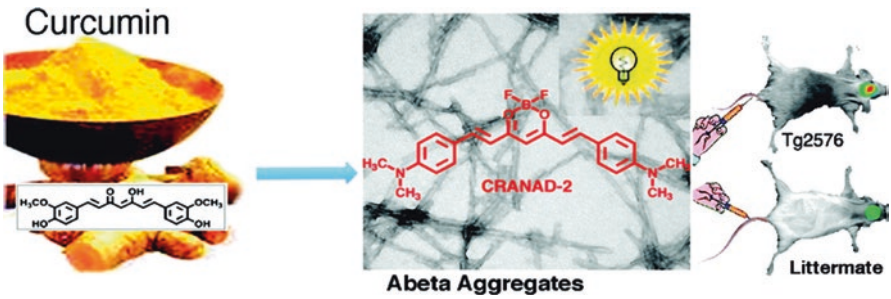

Fig. 9.6 In vitro and in vivo monitoring of AA using CRANAD-2. Reproduced with permission (Ran et al. 2009), Copyrights reserved to American Chemical Society 2009

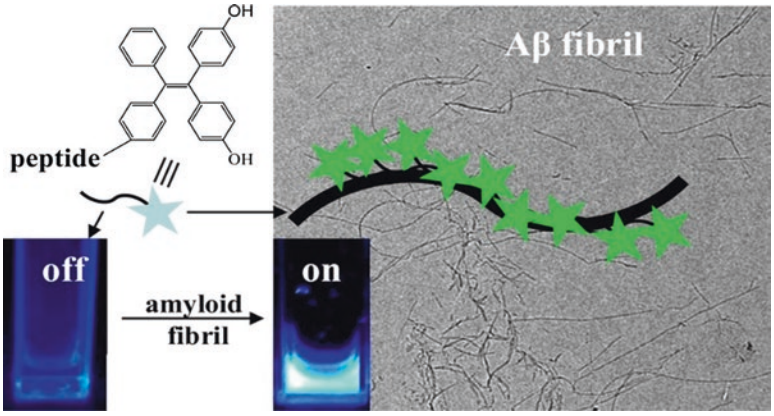

Fig. 9.7 Fluorescence glow of tetraphenylethene peptide on amyloid peptide fibrillation. Reproduced with permission of Pradhan et al. (2015), Copyright reserved to American Chemical Society 2015

amyloids. A new NIR curcumin derivative (CRANAD-2) probe for monitoring Aβ aggregation (Fig. 9.6) was developed. As-prepared NIR probe is highly sensitive and has ability to bind with Aβ aggregates. Similarly, CRANAD-2 can also easily cross the BBB, so it could noninvasively detect the amyloids during in vivo studies (Ran et al. 2009).

Normally used fluorescence molecules upon binding with the amyloid peptide undergo fluorescence quenching. In addition to these fluorophores, there is another class of compounds which are nonfluorescent in their single phase; however, upon aggregation these molecules show glow/fluorescence. This kind of fluorescence is termed as aggregation-induced emission (AIE). Molecules that undergo AIE emission include silole, iridium and platinum metal complexes, arylbenzene, arylethene, and aminobenzoic acid derivatives (Luo et al. 2001; Gao et al. 2009; Hong et al. 2011; Liu et al. 2012; Alam et al. 2014). Later, AIE probes, namely, alkoxy bridged binuclear rhenium (I) complexes and tetraphenylethene peptide (Fig. 9.7), were reported, these probes upon bond formation with amyloid fibrils show AIE that is

detected, and extent of AIE is proportional to concentration of amyloid fibrils (Sathish et al. 2014; Pradhan et al. 2015).

In addition of above-stated methods for the determination of amyloid aggregates, few studies are reported in literature that can determine the main culprits of amyloidosis, i.e., oligomers/monomers. ^{19}FNMR techniques can be used for the AA detection; different intermediate products formed during hIAPP aggregation could be detected by ^{19}FNMR techniques. Extraction and study of associated toxicities of these intermediate products will help to understand hIAPP aggregation mechanism and associated toxicity (Patel et al. 2014). Further, Teoh and co-workers in their work designed a probe BoDipy-Oligomer that can particularly bind with the oligomers (Teoh et al. 2015); apart from these methods oligomer-specific antibody, peptide-based fluorescent protein, ELISA method, and oligomer-specific peptide-Flash system are also reported in literature (Hu et al. 2010; Liu et al. 2012; Morgado et al. 2012; Takahashi and Mihara 2012; Bruggink et al. 2013). Limitation of these methods is that from these reported methods only oligomers can be detected and not the monomeric forms that can lead to fibril formation.

A new multimodal probe for both in vivo and in vitro detection of monomers/oligomers of three different amyloid peptides (hIAPP, Aβ, and insulin) was designed by Yousaf and co-workers (Fig. 9.8a). As-designed bovine serum albumin-coated fluorine functionalized graphene quantum dots (BSA@FGQDs) probe can effectively monitor amyloid monomers/oligomers and fibrils in vitro through fluorescence quenching of BSA@FGQDs. Whenever BSA@FGQDs probe comes in contact with amyloid monomers/oligomer, fluorescence quenching was observed, attributing to the resonance energy transfer between BSA@FGQDs probe and amyloid peptide as well as to the static quenching (Fig. 9.8b). Further, as-prepared BSA@FGQDs possess higher sensitivity than that of conventional dye thioflavin T. Mechanistically, BSA@FGQDs probe interacts with different morphological states of amyloid aggregates confirmed by various microscopic techniques like atomic force microscope and transmission electron microscope, whereas magnetic properties and lipophilic properties of BSA@FGQDs enable it to cross BBB and bind with Aβ plaques with high sensitivity, thereby allowing efficient MRI-based in vivo detection. Moreover, BSA@FGQDs probe not only successfully detected amyloid plaques in brain of AD mice but also enhanced the contrast and enables clear visualization of amyloid plaques at various stages (Yousaf et al. 2018).

After successful detection of amyloidosis next step is designing a suitable and effective treatment methodology. At present, there are a large number of molecular regulators and nanomaterials have been reported that are inhibiting Aβ aggregation. Depending on chemical structure, these molecular inhibitors can be classified into four categories: (1) proteins or polypeptides, (2) modified polypeptides, (3) antibodies targeting amyloid, and (4) small organic molecules. Among them, small organic molecules that include surfactants, copper and zinc ion chelating agents, dye molecules (Congo red, thioflavin T), and small biologically active molecules (apomorphine, rifamycin, curcumin, porphyrin) derivatives. Studying the interaction between these molecules and amyloid greatly enriches the understanding of the conformational folding of biomacromolecules; improves the design efficiency of synthetic artificial antibodies, peptides, and protein-targeted drugs; and contributes

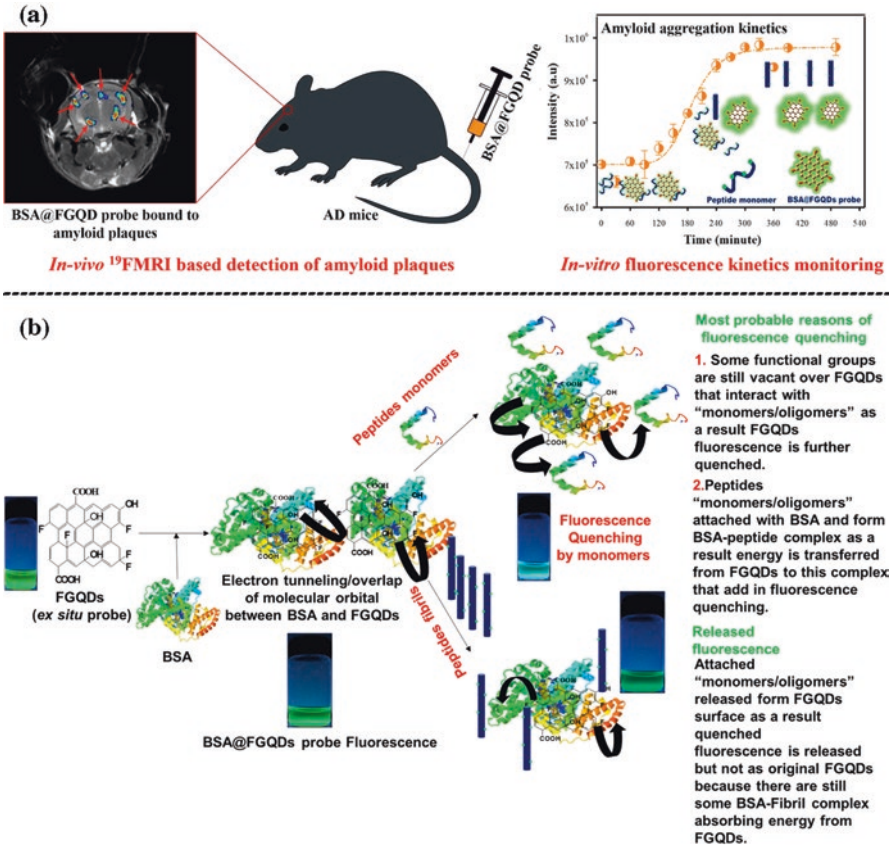

Fig. 9.8 (**a**) An overview of in vivo and in vitro AA detection using BSA@FGQDs. (**b**) Amyloid monomers/oligomer detection mechanism. Reproduced with permission of Yousaf et al. (2019), Copyrights reserved to American Chemical Society 2019

to amyloidosis treatment. Although the reported nanomaterial shows good performance but low biocompatibility, self-aggregation and reduced stability is a big hindrance in their application (Wang et al. 2014; Kao et al. 2015; Pithadia et al. 2016; Sun et al. 2016).

9.3.2 Nanomaterials for Alzheimer Disease and Type 2 Diabetes Treatment

The nanomaterials are gaining special interest in biomedical sciences particularly in amyloidosis treatment in comparison to the bulk materials because of their unique structure and easy tailoring in terms of dimensions, size, and surface chemistry like functional groups, hydrophilicity, and hydrophobicity. Therefore, exploring the

interaction of amyloid peptide and proteins with the nanomaterials is the major focus of current research. Moreover, large surface-to-volume ratio of NPs makes them potential material to be studied to explore the effect of different surfaces on process of AA. Up till now extensive research is done on NPs as anti-amyloidogenic agent, e.g., gold NPs, dendrimers, polymer NPs, inorganic semiconductor QDs, magnetic NPs, and carbon materials (fullerenes, carbon nanotubes, and graphene). These nanomaterials could either enhance or halt process of AA and it depends upon chemical structure, surface area, concentration, and physicochemical properties. Hence, performance of anti-amyloidogenic agents can be controlled by controlling above-stated parameters (Zhang et al. 2013a).

Aβ aggregates could be detected by superparamagnetic iron oxide NPs (SPIONs); interaction between SPIONs and Aβ depends upon extent of physical and chemical properties such as coating, valence state, and concentration of SPIONs (Mahmoudi et al. 2013). Further, *N*-acetyl-L-cysteine coated quantum dots (NAC-QDs) can significantly inhibit the fibrosis of Aβ by limiting nucleation and production processes. It is proposed that the hydrogen bond interaction between Aβ and NAC-QDs mainly cause inhibition of fibrosis by NAC-QDs (Xiao et al. 2010). Similarly, N-isopropylacrylamide/N-tert-butylacrylamide (NiPAM/BAM) copolymer NPs can also inhibit the Aβ aggregation by occupying Aβ monomer or oligomerization sites (Cabaleiro-Lago et al. 2008). The binding site on the body, which in turn affects the nucleation process of Aβ, hinders the aggregation process. Studies have shown that the fluorinated core-shell structure γ-Fe_2O_3/poly(2,2,3,3,4,4,4-heptafluorobutyl acrylate) (γ-Fe_2O_3/PHFBA) also has an inhibitory effect on insulin protein fibrosis. In the presence of γ-Fe_2O_3/PHFBA NPs, insulin eggs will remain in the alpha-helical structure for a long time, delaying aggregation. However, γ-Fe_2O_3 did not inhibit insulin protein because of the hydrophobic interaction generated by the fluorinated functional groups (–CF_2 and –CF_3) on the surface of γ-Fe_2O_3 to stabilize the α-helical structure (Skaat et al. 2009).

Beside metallic inhibitors, carbon nanomaterials are an emerging class of materials which is quite suitable for living beings because of their high biocompatibility than other nanomaterials as discussed before as well. Among carbon nanomaterials, two-dimensional graphene oxide sheet (GO) is a rising star and could be used as an anti-amyloidogenic agent. GO has been used an anti-amyloidogenic agent for hIAPP aggregation inhibition; GO efficiently inhibited hIAPP aggregation and significantly reduced its toxicity level. GO inhibited aggregation through hydrophobic, electrostatic, and aromatic stacking interaction with hIAPP. These interactions were confirmed from molecular dynamics simulations that GO can bind with hIAPP and prevented self-association of hIAPP (Nedumpully-Govindan et al. 2016). Similarly, another class of carbon nanomaterials is zero-dimensional graphene quantum dots (GQDs); just like other carbon materials GQDs possess high biocompatibility, rich surface chemistry, high water dispersibility, good stability, and larger surface area that can be easily tailored to enhance physicochemical properties to make it a potential anti-amyloidogenic theranostic agent (Zhang et al. 2013b; Zhou et al. 2016). GQDs have been used in drug delivery and now application of GQDs in the field of peptide aggregation regulation has just begun. Till now, inhibitory potential of

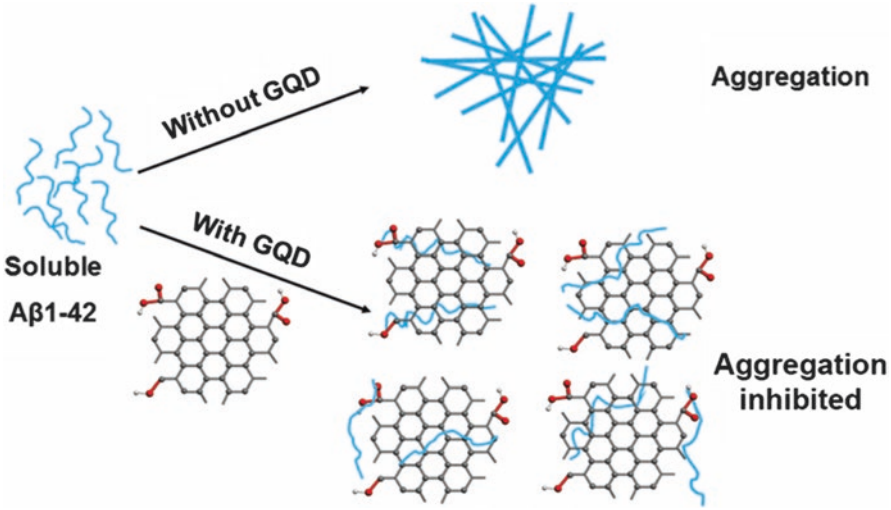

Fig. 9.9 Inhibition of aggregation of $A\beta_{1-42}$ by GQDs. Reproduced with permission of Liu et al. (2015), Copyright reserved to Royal Society of Chemistry, 2015

GQDs for a single peptide, i.e., Aβ (Fig. 9.9), is reported only. The inhibitory effect of GQDs was based on surface electronegativity of GQDs. In these findings, it is also demonstrated that the GQDs interact with hydrophobic motif of Aβ and prevents its self-aggregation into amyloid deposit, which ultimately prevents the disease propagation (Liu et al. 2015). Recently, fluorinated graphene quantum dots (FGQDs) with enhanced properties than GQDs are fabricated. FGQDs possess strong ability for in vitro inhibition of hIAPP through hydrophobic and electrostatic interaction with hIAPP peptide, which inhibits hIAPP aggregation and reduced hIAPP induced cytotoxicity level as shown in Fig. 9.10 (Yousaf et al. 2017).

9.3.3 Nanocarriers for Alzheimer Disease Treatment

Neurological disorders occurred during AD are because of Aβ oligomers and free radicals. It is found that the nanomaterials could act as a protective material and prevent the aggregation and formation of Aβ oligomers and reduce oxidative stress of free radicals. Moreover, major hindrance in AD treatment is because of limited access of drug to the target of action and inability to cross BBB due to variation in body conditions (pH and temperature). Moreover, drug is broken down before reaching its destination resulting in quick elimination of drug from body due to reduced half-life. Sometimes, enhanced interaction of drug with nonspecific sites leads to vomiting, abdominal pain, and nausea. Drug mode of action and efficiency can be enhanced by targeted drug delivery by using nanocarriers; these carriers enhanced the therapeutic potential of the drug by improving its physicochemical

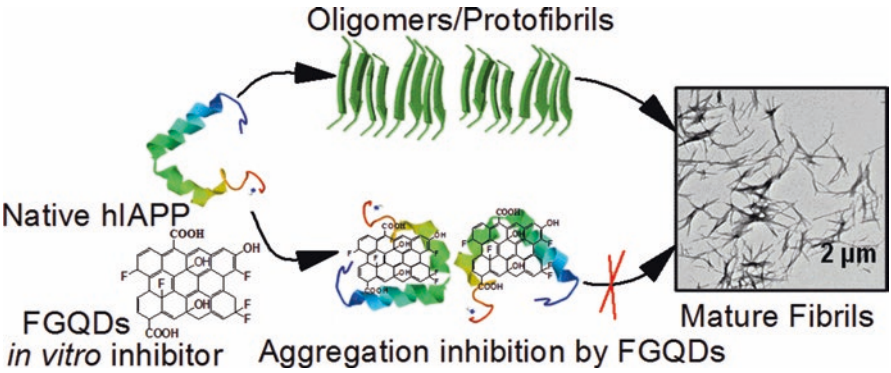

Fig. 9.10 hIAPP aggregation inhibition by FGQDs. Reproduced with permission of Yousaf et al. (2017), Copyright reserved to American Chemical Society 2017

properties like molecular weight, polarity, solubility, net charge, affinity for hydrophobic, or lipid moieties. Depending upon the nature of nanocarrier, these are classified into organic (liposomes, emulsions, polymers, dendrimers, and solid-lipid NPs) and inorganic (silica, gold, and carbon). Mode of action adopted by NPs is summarized in Fig. 9.11.

9.3.4 Nanotheranostics for Alzheimer Disease and Type 2 Diabetes Treatment

Combing diagnosis and therapeutic strategy into a signal term "theranostics" is the focus of current researchers. Different types of amyloid theranostics reported so far in literature are curcumin, rosmarinic acid, polyphenolic molecules, tannic acid, and their derivatives (Yang et al. 2005). These materials show remarkable performance as a theranostic agent; they inhibit AA through π-π stacking interactions between the phenolic rings of the polyphenols and the aromatic amino acid residues in the amyloid protein. Other structural units form hydrogen bonds to stabilize the polyphenolic molecule-protein complex. Zhang et al. (2013a, b) synthesized NIR-based curcumin derivative (CRANAD-17) that could be used as a theranostics. A CRANAD-17 possesses ability to break crosslinking of Aβ induced by copper and could bind with soluble and insoluble forms of Aβ (Zhang et al. 2013b). Moreover, Li et al. (2016) synthesized charged molecules; among them DBA-SLOH molecule excellent performance allows efficient NIR-based detection of amyloid plaques by crossing BBB of AD mice.

High biocompatibility and lipophilicity of (E)-4-(4-(dibutylamino)styryl)-1-(2-hydroxyethyl)quinolin-1-ium chloride (DBA-SLOH) molecule was due to addition of lipophilic alkyl chains. At the same time, DBA-SLOH molecule showed binding affinity for both monomer and aggregates of Aβ, inhibiting the aggregation of

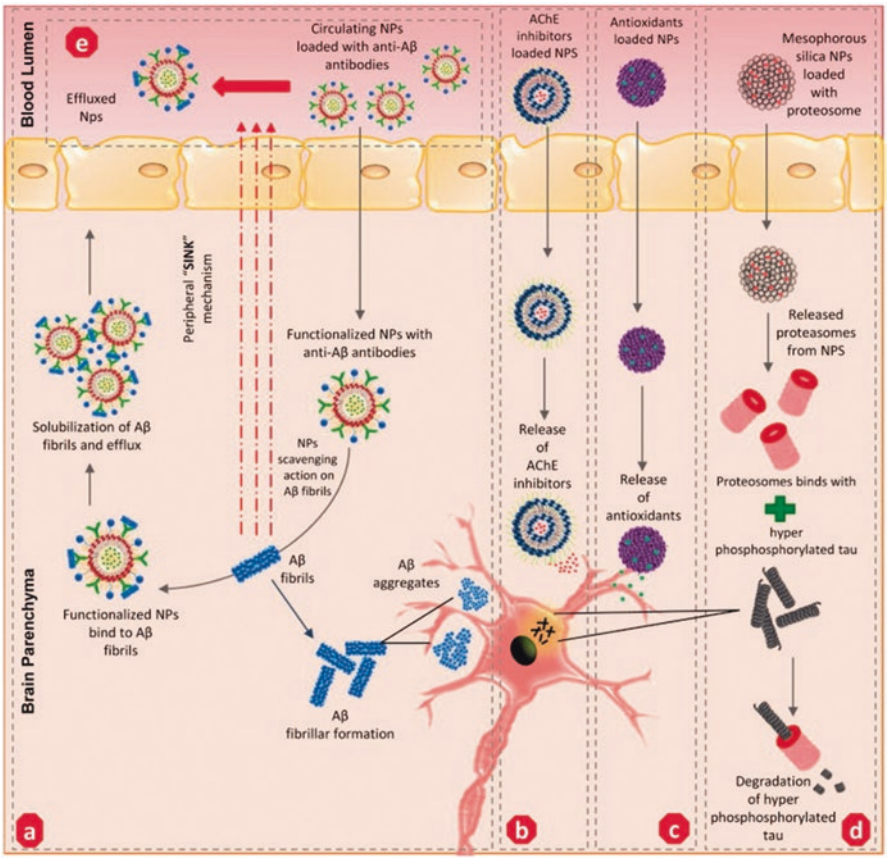

Fig. 9.11 Nanocarrier mode of action for the treatment of AD. (a) Modulation of Aβ fibrils through anti-Aβ functionalized NPs, (b) treatment of cholinergic system impairment using acetylcholinesterase inhibitor/NP conjugate, (c) oxidative stress milieu treatment by antioxidant/NP conjugate, (d) treatment/targeting of hyper-phosphorylated tau proteins using proteasomes/NPs, (e) initiation of "sink mechanism" by anti-Aβ/circulating NPs for capturing Aβ fibrils from brain and taking it to the blood circulation (effluence). Reproduced with the permission of Karthivashan et al. (2018), Copyright reserved to Taylor and Francis Group, 2018

amyloid peptide monomers to avoid generation of toxic oligomers ultimately showing a great potential as theranostic agent for detection of AA (Li et al. 2016). In addition to abovementioned NIR probes, phenothiazine-based compounds can also act as a theranostic for Aβ aggregation both in vivo and in vitro. Donor acceptor like architecture of phenothiazine-based enables it to inhibit and detect AA. Upon binding with amyloid fibrils fluorescence intensity of probe was enhanced; moreover, this probe showed high stability in mouse serum and low toxicity for human neuronal cells (Dao et al. 2017).

A new fluorescence-based theranostic chelator (BTTA) could specifically target metal-Aβ aggregates. BTTA efficiently detects the metal-Aβ aggregates both in vivo

and in vitro by variation in fluorescence, and attenuation of aggregation is confirmed form reduced neurotoxicity (Yang et al. 2016). Along with the fluorescence-based theranostics, MRI detection-based theranostic agents have also been reported. Furthermore, lipophilic molecules analogous to the Congo red (CR) are available as amyloid theranostics; one of them contains ^{19}F as MRI active agent. Just like CR these molecules possess the ability to bind amyloid plaques and could noninvasively detect amyloid plaques in brain of living mice (Skovronsky et al. 2000; Higuchi et al. 2005).

Just like smaller molecules, a variety of nanomaterials can be used as amyloid theranostic agents because of their unique biological and physicochemical properties. Magnetic SPIONs have been reported to be used as amyloid theranostic agents (Amiri et al. 2013; Sillerud et al. 2013). Although all these materials showed remarkable performance, their high level of toxicity is a big hurdle in their application as a theranostic material that should be considered carefully, and better solutions should be proposed.

9.4 Conclusions

In summary, a fast evolution of AD as a major public health concern in the last few years demands better methodologies to understand this neuropathology in better way. Therefore, it is crucial first to perceive the genesis and the nucleation and aggregation of Aβ fibrils and plaques in better way, which appears before the apparition of the symptoms. Thus, a number of macroscopic methods based on conventional medical imaging techniques such as MRI, or NIR, imaging and microscopic systems based on two-photon excited or electron microscopy are developed for early detection of Aβ fibrils and plaques. These methods allow us to better understand the disease evolution and help us in developing better treatment methods.

It's understandable that timely diagnosis of AD development could prevent uncontrollable and irreversible damage of AD. But designing of a reliable and cost-effective strategy is a big challenge in current scenario; however, utilizing nanodiagnostic tools and nanocarriers would solve this problem. Currently available nanodiagnostic tool can detect amyloid biomarker either in vitro or in vivo depending upon physicochemical interaction with amyloid protein. On the other side, nanocarriers have enhanced the targeted drug delivery to specifically improve the AD treatment. Although therapeutics has been approved by food and drug administration (FDA) and is available for amyloidosis treatment, development of nanodrugs will enhance working efficiency of drugs, but stability, toxicity, and desired drug release from NPs are needed to be addressed strictly to design a reliable and cost-effective method. Despite extensive research over amyloidosis treatment and diagnosis, still there is a space to design highly biocompatible materials; in this regard carbon-based nanomaterials will be a good option because of their low toxicity compared to other metallic, polymeric, and inorganic nanomaterials.

Acknowledgments The authors would like to acknowledge the Vice-Chancellor Fellowship Scheme at RMIT University and University of Agriculture, Faisalabad, Pakistan, the RMIT MicroNano Research Facility (MNRF) in the Victorian node of the Australian National Fabrication Facility (ANFF), and the RMIT Microscopy and Microanalysis Facility (RMMF) for their support in this work.

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Chapter 10
Applications of Nanomaterials in Bone Tissue Engineering

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Abstract Nanobiotechnology has emerged as a promising field that holds enormous potential in modern medicine. The use of such nanosized particles for delivery of drugs, nucleic acids and fabrication of scaffolds has revolutionised the methods of tissue engineering. The in vitro and in vivo properties of nanoparticles are largely governed by their size, shape, charge and surface topography. These particles hold a great potential with respect to targeted drug delivery, antibacterial properties, ability to adhere to variety of molecules and cellular uptake, due to large surface area to volume ratio. The application of nanoparticles for bone tissue engineering has gained popularity because their small size enables them to travel through highly compact microarchitecture of bone tissue and cross the blood-bone barrier. Additionally, nanoparticles play an important role in enhancing the cellular adherence of the scaffolds used for providing support to the regenerating bone. The present chapter provides a comprehensive overview of chemically synthesised organic and inorganic nanoparticles specifically with respect to bone tissue engineering.

Keywords Nanoparticle · Implant · Osteoinductive · Tissue regeneration · Hydroxyapatite · Bone

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10.1 Introduction

Bone is a complex hierarchical structure where the interplay of organic and inorganic mineral phases at different length scales affects its functional and physicochemical properties. Mineralisation of bone matrix involves a series of complex events carried and regulated by osteoblasts, ultimately leading to deposition of an inorganic mineral phase. The later stages of osteoblast differentiation are associated with nucleation of hydroxyapatite (HA) nanocrystals in an organic collagenous matrix, forming a heterogeneous tissue. Many reports suggests association of nanohydroxyapatite (nHAp) with increase in both gene expression profiles of bone-specific markers and protein deposition, during osteogenic differentiation of mesenchymal stem cells (MSCs) (Campi et al. 2017).

It has been found that bone injuries create gap and cause the accumulation of necrotic bone debris. Repetitive loading results in accumulation of microdamage and diffused damage in the case of clinical fractures. Fragility fractures have been observed in elderly, whereas active individuals mainly suffer due to fatigue fractures. These phenomena are also suggested to be an outcome of long-term antiresorptive treatments for osteoporosis (Ross and Roeder 2011; Li et al. 2016c). It has also been found that as the mineral components of bone degrade, a bone crack is generated that releases ions with different diffusion coefficients, resulting in generation of an electric field pointing outwards. This gradient of ions serves as detection probe for bone cracks and also attracts the negatively charged moieties such as bisphosphonates (Li et al. 2016b). Migration of inflammatory cells from this damaged site triggers a cascade of both inflammatory and bone building signals that alleviate the migration of phagocytic cells. Ultimately, the healing cascade is initiated by removal of necrotic tissue and propagation of vascularisation (Ross and Roeder 2011; Li et al. 2016c). Macrophages regulate this process of wound healing and tissue regeneration by secretion of various cytokines including interleukins, chemokines, growth factors and other small molecules. However, when a tissue defect advances beyond a critical size, the natural process of healing does not ensure complete recovery and require implantation of scaffolds for guided tissue regeneration. But usually the scaffolds invite foreign body reaction initiated by macrophages that eventually cause inflammation (Hao et al. 2017). Therefore, bone scaffolds are usually used to analyse immunogenicity under two sets of conditions "macrophage only" and "macrophage and osteoblast" to mimic the cellular interactions (Behera et al. 2017). Therapies for guided tissue regeneration are mostly aimed at producing a new bone that supports the synergistic processes of osteoinduction and osteoconduction (Ross and Roeder 2011; Li et al. 2016c).

Till date autologous bone grafts have been a preferred treatment for large- and critical-sized bone defects. But requirement of two separate surgical operations with risk of wound dehiscence, infection, vessel injury, donor site morbidity and hematomas are few of the major challenges associated with autologous implantation. However, the difficulties of creating modifications that match the defect

morphologies enhance the possibilities of graft versus host diseases (GvHD) (Wang et al. 2013; Kim et al. 2016; Ding et al. 2017). The alternative approach of using allografts also has limitations, including higher cost, greater variation in clinical results, viral transmission, risk of infection and immunogenicity (Kim et al. 2016).

The methods used for treatment of diaphyseal fractures involve the use of bone plates to restrain the movement of the fragments. The conventional compression plates used for such treatments are made up of metals such as stainless steel, titanium and alloys of cobalt-chromium. The elastic modulus of these metal plates is 5–10 times higher than that of cortical bone that causes stress shielding and fracturing of bones after the removal of plate. Stress shielding is caused due to the absence of load on the healing bone because of transfer of all the stress on the metal plate (Aydin et al. 2011). Moreover, these metallic plates undergo a very slow degradation (almost no degradation), and therefore they hinder the formation of new bones during late stages of bone repair. In addition to the metal implants, the drugs used for the regenerative approaches are limited in their action due to rapid clearance from the body even before they fully affect their target sites. To combat this limitation, drugs are generally administered frequently and/or in high dosages. But such administration schedules have been reported to cause detrimental side effects. Therefore, there is a strong need to develop a targeted delivery system for a sustained (over an extended period because in the immediate aftermath of the surgery growth factors are present in abundance) and specific release (controlled dosage to prevent possibility of side effects) of drug to the bone.

As bone possesses one of the most complex hierarchical micro- and nanoarchitecture in the human body, the delivery devices should allow enough exposure of the drug to the mineralised structure of bone before it gets excreted (Jiang et al. 2014). Localised delivery of appropriate growth factor or biomolecules can be achieved (1) by incorporation of biomolecules within biodegradable particulate carriers (nanoparticles) and/or (2) by inclusion of such particulate carriers into suitable porous scaffolds (Nandagiri et al. 2011). The fabrication of scaffolds incorporating nanoparticles involves the use of three major technologies, namely, freeze-drying, electrospinning and hydrogels. All these processes involve preparation of a homogeneous solution of specified concentrations of polymers, proteins and/or materials/nanoparticles.

1. Freeze-drying method employs overnight freezing at −80 °C and lyophilisation at temperature ranging from −50 to −80 °C, till the solution is completely dried. The scaffolds obtained are further cross-linked using cross-linking agents such as glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)-N-hydroxysuccinimide (NHS) and lyophilised again.
2. For electrospinning, the homogeneous mixture is loaded into a syringe, and the mixture is electrospun at a flow rate of 0.5–1 ml/h at a voltage of 12–20 kV, at a distance of 12–20 cm from the collector plate. The electrospun scaffolds are then dried either by lyophilisation or by dry heating.

3. In order to combat the limitations of slow rate of fibre production in electrospinning, air spray spinning method has been developed. In this method, pressurised gas at extremely low velocity is used for stretching of polymer solutions into thin fibres at the nozzle outlet.
4. For synthesis of hydrogels, the homogeneous mixture is incubated at a particular temperature for a specified time for gelation and then immersed into a suspension of nanoparticles.

Therefore, nanoparticulate systems have attracted particular interests in the field of regenerative medicine, because of the following reasons: (1) relatively high drug encapsulation capacity, (2) compatibility with various routes of administration (especially intravenous), (3) protection of drugs from degradation, (4) improvement of transmucosal transport of drugs and (5) controlled release of encapsulated drugs (Jiang et al. 2014).

Nanoparticles with various shapes and dimensions can be generated using various physico-chemical and biological routes. These techniques would involve either a top-down (breaking down of a bulk material into smaller pieces using mechanical, chemical or other form of energy) or bottom-up (to synthesise the material from atomic or molecular species via chemical reactions that allow the precursor to grow in size) approach (Table 10.1). Chemical synthesis of nanoparticles has a glorious history, especially because of its extensive use in production of metal and metal-oxide nanoparticles. These chemically synthesised nanoparticles however have been implicated in cellular toxicity. Moreover, the method of synthesis is expensive, not environment-friendly and utilises non-renewable materials, chemical derivatives and stoichiometric reagents. This has made the use of biological sources as the preferred alternatives. The goal of the green nanotechnology is to use whole biological organisms or their parts (molecules, cells, tissues or organs) as raw materials to develop novel and valuable nanomaterials with sustainable benefits. Although green processes are regarded as harmless, inexpensive, sustainable and biocompatible, they involve a time-consuming process of synthesis, challenges associated with handling of microbes and limitations in providing improved features over dimension distribution, form and crystallinity. Both these types of nanoparticles are either used as drug carriers or are incorporated into biodegradable scaffolds for guided tissue regeneration (Gross 2015).

Nanoparticles can be classified into two major categories, namely, inorganic and organic (or natural materials) nanoparticles. Carbon nanoparticles/carbonaceous substrates comprise for organic nanoparticles. Metal and metal oxide nanoparticles fall under the category of inorganic nanoparticles. Hybrid organic-inorganic materials form a separate category of nanoparticles, where a combination of an inorganic component and an (bio-)organic component is synthesised at nanoscale. The property of the hybrid material may be distinctly different to that of the individual components (Guo et al. 2018). In this chapter we have discussed chemically synthesised inorganic and organic nanoparticles with special emphasis on their applicability in bone tissue engineering (Gross 2015).

Table 10.1 Methods for synthesis of nanoparticles

Nanoparticle	Method of synthesis
Magnesium/ magnesium oxide	Hydroxide precipitation method (nMgO) (Suryavanshi et al. 2017)
Zinc	Flame pyrolysis method (nZnO) (Memarzadeh et al. 2015)
Iron	Chemical co-precipitation method (γIONPs) (Xia et al. 2018a)
	Co-precipitation method under ultrasound condition (magnetite nanoparticles) (Aliramaji et al. 2017)
	Modified chemical co-precipitation method (Fe_3O_4/PLLA nanoparticles) (Shan et al. 2013)
	Ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) in 1 M HCl and ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), mixed at room temperature, precipitated by 1.5 M NaOH. The nanoparticles coated with citric acid via the constituent COOH groups (Singh et al. 2014)
	Chemical co-precipitation method using ferrous chloride hexahydrate and ferrous chloride tetrahydrate for synthesis of IONPs. IONPs coated with polyglucose-sorbitol-carboxymethylether (PSC). The mixture heated at 80 °C for 1 h and purified with five cycles of ultrafiltration against deionised water using a 100 kDa membrane (average size 30.15 ± 0.1843 nm) (Wang et al. 2016a)
	Ferric chloride hexahydrate and ferrous chloride tetrahydrate dissolved in distilled water with vigorous stirring in nitrogen atmosphere at ambient temperature. Aqueous ammonium hydroxide and oleic acid (OA)/citric acid (CA) added into the mixture at 80 °C, 1 h. Aqueous solutions freeze-dried and the resulting products stored at 4 °C for further use (Huang et al. 2011)
	Colloidal magnetite added to a lipid mixture consisting of N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG, a cationic lipid), dilauroylphosphatidylcholine (DLPC) and dioleoylphosphatidylethanolamine (DOPE) in 1:2:2 molar ratio. Average particle size of magnetite nanoparticles (Fe_3O_4) is 10 nm and that of magnetite cationic liposome is 150 nm (Ho et al. 2007)
Silicon	Tetraethyl orthosilicate (TEOS, 99.90% pure) in absolute ethanol and calcium nitrate tetrahydrate in distilled water, mixed for 3 h after addition of citric acid monohydrate (99%) and added drop by drop to distilled water maintaining the pH at 11.5. The precipitate stirred for 48 h followed by freeze-drying. The BG nanoparticles were calcined at 700 °C for 3 h (Luz and Mano 2012)
	Cetyltrimethylammonium bromide (CTAB) and ammonium fluoride (NH_4F), dissolved in distilled water and stirred for 1 h at 80 °C, tetraethoxysilane added dropwise. Supernatant collected and centrifuged after ageing overnight at room temperature (mesoporous silica nanospheres) (Kim et al. 2013; Shi et al. 2016)
	Rice husk fired at 700 °C for 3 h in muffle furnace, the ash washed with deionised water followed by acid leaching with 6 N HCl for 1 h to remove the impurities. Washed rice husk boiled with sodium hydroxide for 1 h with continuous stirring, sodium silicate extract obtained in liquid form. pH reduced to 2 with concentrated sulphuric acid to obtain silica precipitates. The precipitate dried in an oven at 100 °C for 12 h and the powder further calcined at 500 °C for 3 h in the furnace (Suriyaprabha et al. 2015)
	CTAB and $NH_3{\cdot}H_2O$, mixed in double-distilled water and then stirred for 15 min at 60 °C. Next, tetraethyl orthosilicate and calcium nitrate, added with vigorous stirring for 3 h. The precipitated products collected by filtration and washed with hydrochloric acid and ethanol. The powders dried at 60 °C overnight and sintered at 800 °C for 2 h to remove the remaining traces of CTAB (Huang et al. 2017)

(continued)

Table 10.1 (continued)

Nanoparticle	Method of synthesis
Bioactive glass	Sequential reagent dissolution resulted in hydrolysis and polycondensation reactions. Tetraethyl orthosilicate was used as silicon precursor, ammonium phosphate dibasic as phosphorus precursor, calcium nitrate tetrahydrate as calcium precursor, citric acid monohydrate to promote hydrolysis, absolute ethanol and ammonium hydroxide as gelling agent and polyethylene glycol (PEG) as the surfactant. The BG-NPs sintered at 700 °C for 5 h. Spherical particles with composition $SiO_2/CaO/P_2O_5$ (mol.%) = 55:40:5 and size <50 nm were obtained (Luz et al. 2012; Mota et al. 2012; Tavakolizadeh et al. 2017)
Aluminium	The nanoAl_2O_3 was produced from raw bauxite by adding sodium hydroxide for aluminium hydroxide formation followed by mineral acid precipitation at pH 7 to obtain the precipitate. The white precipitate of aluminium hydroxide was obtained after ageing at 80 °C for 12 h followed by incubation at 120 °C for 1 h that was further calcined at 500 °C for 6 h (Suriyaprabha et al. 2015)
Silver	Mixing of silver nitrate ($AgNO_3$) with silk fibroin solution, to prepare hydrogels with AgNPs (Ribeiro et al. 2017)
Gold	Gold nanoparticles were synthesised by reducing gold ions in silk fibroin solution. Gold (III) chloride trihydrate ($HAuCl_4{\cdot}3H_2O$) mixed with solution of nanohydroxyapatite in ethanol, followed by slow mixing with the silk fibroin solution to get hydrogels with AuNPs at 37 °C (Ross and Roeder 2011; Zhang et al. 2014; Ko et al. 2015; Heo et al. 2016; Lee et al. 2016a; Ribeiro et al. 2017)
	AuNPs were synthesised by the reduction of chloroauric acid with trisodium citrate (Heo et al. 2017a)
	Ultrapure deionised water and chloroauric acid trihydrate mixed and solution heated to its boiling point. Then, sodium citrate added to the solution and mixture heated for another 25 min, subsequently cooled down to room temperature, to obtain AuNPs (Xia et al. 2018b)
	$HAuCl_4$ heated to 100 °C under constant stirring, sodium citrate solution added to the hot gold solution. The mixture refluxed for 15 min and rapidly cooled in an ice bath at 15 °C to obtain AuNPs (Tentor et al. 2017)
Hydroxyapatite (HA)	Wet chemical precipitation followed by hydrothermal treatment for crystallisation of nano-HA (Hickey et al. 2015; Nga et al. 2015)
	nHA particles synthesised by wet precipitation between calcium hydroxide ($Ca(OH)_2$) and orthophosphoric (H_3PO_4) acid (Aydin et al. 2011; Abdal-Hay et al. 2013; Pan et al. 2014; Memarzadeh et al. 2015; Shakir et al. 2016; Yu et al. 2018)
	Calcium nitrate tetrahydrate (CNTH) dissolved in water and ethanol (1:12 by volume) mixture and introduced into the reaction flask. pH of the solution maintained in the range of 10–12 by slow addition of ammonia solution. Aqueous solution of diammonium hydrogen phosphate was added with vigorous stirring for 4 h at 40 ± 2 °C. After the completion of the reaction, the product was filtered, washed and dried at 40 °C for 14 h (Nazeer et al. 2017)
	Aqueous solutions of $Ca(OH)_2$ and H_3PO_4 were prepared separately. The fresh silk fibroin solution was incubated at 60 °C for 24 h to generate homogeneous nanoparticles. H_3PO_4 solutions were first mixed with silk fibroin solution to form a silk fibroin-H_3PO_4 mixed solution that was dropwise added to suspension of $Ca(OH)_2$ with vigorous stirring. The solution heated at 70 °C and the pH adjusted to ~9 with NaOH. The emulsions centrifuged and washed gently with distilled water to recover the silk fibroin-coated HA nanoparticles (Ding et al. 2017)

	NaCl, KCL, $CaCl_2{\cdot}2H_2O$, $MgCl_2{\cdot}6H_2O$ and $NaH_2PO_4{\cdot}H_2O$, dissolved in distilled water under stirring. $NaHCO_3$ was added to solution prior to HA precipitation from simulated body fluid solution. The suspension then centrifuged and washed with ethanol and distilled water to remove impurities. The powders left overnight in incubator to dry Boric acid was dissolved in distilled water prior to salt addition and pH adjusted to between 6.5 and 7.4 by adding NaOH. HA was synthesised as mentioned above (Boron doped HA particles) (Arslan et al. 2018)
	Sol-gel method; $Ca(NO_3)_3{\cdot}4H_2O$ and $NH_3{\cdot}H_2O$, dissolved in anhydrous ethanol, pH 10, that contains PEG to form solution 1. Then, $(NH_4)_2HPO_4$ added into H_2O to form solution 2. Solution 2 was introduced into solution 1. After vigorously stirring for 2 h at 25 °C, the obtained collosol was transferred into a water bath kettle and maintained at 60 °C for 24 h. The precipitates separated by centrifugation, washed with deionised water and followed by ethanol, dried in air at 100 °C to obtain the intermediate products. The obtained products were heat treated at 800 °C for 2 h to obtain the HA nanoparticles (Zhou et al. 2017)
	Sonochemistry method; eggshells of boiled eggs collected and adhered organic matter eliminated. They were crushed and washed with deionised water. After washing, the eggshells were ultrasonicated in deionised water and H_2O_2 for 1 h, washed with deionised water and dried in oven for 2 h at 100 °C. After drying, the eggshells were ground and ball milled to obtain a fine powder of nanoparticles. Furthermore, the eggshells were heated at 200 °C and calcined at 900 °C for 2 h in a box furnace to decompose organic matter and to convert the calcium carbonate to calcium oxide. The calcium oxide obtained from eggshells was weighed and dispersed in deionised water and ammonium hydroxide and ultrasonicated for 1 h to form CaOH (Ingole et al. 2017)
	Calcium sucrate solution was prepared by dissolving CaO in sucrose solution; Triton X-100 added to this solution while stirring in order to form micelles. Ammonium dihydrogen orthophosphate was added dropwise to the calcium sucrate solution. The mixture was then stirred for 12 h, and the suspension thus obtained was collected by centrifugation. The collected HA particles were washed with distilled water in order to remove Triton X-100 and were dispersed in water. HA dispersion was diluted using distilled water, and it was used as feed suspension to fabricate HA on surface-modified titanium metal surfaces using atomised spray pyrolysis technique (Rajapakse et al. 2016)
	Aqueous precipitation reaction using silk as template and surface stabiliser; aqueous solutions of $Ca(OH)_2$ and H_3PO_4 were prepared by dissolving them separately in distilled water. The fresh silk solution (7 wt%) was incubated at 60 °C for 24 h to get homogeneous nanoparticles. Typically, H_3PO_4 solutions were first mixed with silk fibroin solution to form silk fibroin-H_3PO_4 mixed solutions, and then the mixed solutions were dropwise added into $Ca(OH)_2$ suspensions with vigorous stirring. The solution was heated in a water bath at 70 °C, and the pH of the solution was adjusted with NaOH. Finally, the emulsion solutions were centrifuged and washed gently with distilled water (HA/silk core-shell nanoparticles) (Huang et al. 2015)
	Hydrothermal method; a solution of CTAB surfactant was added to a solution of $K_2HPO_4{\cdot}3H_2O$ and mechanically stirred for 20 h at room temperature. $Ca(NO_3)_2$ solution was added to the above mixture, and the resulting solution was thermally treated at 155 °C for 15 h. The material thus obtained was filtered, washed with distilled water and dried at 70 °C, followed by calcination at 680 °C (Maia et al. 2016)
	Aqueous calcium nitrate ($Ca(NO_3)_2$) solution was added to the basic solution of ammonium phosphate ($(NH_4)_3PO_4$) at 50 °C and stirred for 1 h. The solution was then subjected to a heat treatment for 1 h at 100 °C. The obtained gel was then subjected to freeze-drying over a period between 1 to 4 h (pure HA powder) (Ignjatović et al. 2016)

(continued)

Table 10.1 (continued)

Nanoparticle	Method of synthesis
Graphene	Hummer's method; the graphene oxide nanoparticles were obtained by oxidation of graphene nanoplatelets with H_2SO_4, KNO_3 and $KMnO_4$. A mixture of graphene nanoplatelets and KNO_3 in H_2SO_4 was stirred in an ice bath, followed by slow addition of $KMnO_4$, without exceeding the temperature of 20 °C. The temperature was later raised to 35 °C and the mixture was stirred for 1 h. After completion of the reaction, deionised water was gradually added to the solution, and further, mixture of H_2O_2 and water was added. The resulting oxidation causes a colour change of solution from dark purplish green to dark brown. The oxidation reaction was stopped by addition of H_2O_2 solution, which causes high oxidation of graphite and a colour change to bright yellow. The graphene oxide nanoparticles were separated from the mixture by filtration and washed with deionised water, till a pH 4–5 was attained. The washing causes exfoliation of graphene oxide, resulting in thickening of solution, and forms graphene oxide gel (Raucci et al. 2017)
Silk fibroin	Silk fibroin fibres obtained from *Bombyx mori* were dissolved in a ternary solvent (calcium chloride, ethanol and water) at 85 °C for 4 h. This solution was dialysed in distilled water for 72 h using dialysis cellulose tubular membranes to remove the salts, after which the solution was centrifuged at 3000 rpm for 10 min to remove insoluble impurities. The final concentration of the resultant silk fibroin solution (~2.3 wt%) was determined by weighing the remaining sponge weight after freeze-drying. Reverse dialysis against PEG at room temperature is used to increase the concentration of silk fibroin (Kim et al. 2017)
	PCL, PCL/silk fibroin, PCL/silk fibroin/ascorbic acid and PCL/silk fibroin/ascorbic acid/dexamethasone solution were prepared in 1,1,1,3,3,3-hexafluoro-2-propanol. All the polymeric composite solutions were stirred magnetically overnight at room temperature to ensure homogeneity. The solution was then loaded into a syringe with a constant flow rate of 1 ml/h and voltage of 16–17 kV. During the electrospraying process, the particles are collected in an aluminium foil-wrapped collector plate (Gandhimathi 2015)
	Dissolution method; silk fibroin solution was added dropwise to acetone. The precipitated silk fibroin was then collected using centrifugation, and the pellet was then dispersed in deionised water by sonication for 15 min. The nanoparticles were then freeze-dried until further use (Hassani Besheli et al. 2017)
Calcium phosphate	$Na_2HPO_4{\cdot}12H_2O$ and PLA-block-monomethoxy (PEG) (PLA-mPEG) were dissolved in deionised water to obtain PO_4^{3-} ion-containing solution. $CaCl_2$, PLA-mPEG and ammonia (25%) were also dissolved in deionised water to yield Ca^{2+} ion-containing solution. The Ca^{2+} ion-containing solution was slowly added to PO_4^{3-} ion-containing solution and stirred for 1 h at room temperature, to yield amorphous calcium phosphate nanoparticles that were collected by centrifugation. These nanoparticles were then washed with deionised water and ethanol and kept in acetone solution (Fu et al. 2016)
	Dropwise addition of calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) solution to ammonium phosphate dibasic ($(NH_4)_2HPO_4$) solution by a syringe pump, at 55 °C, pH 9.5 for 30 min under stirring, is used to synthesise biphasic calcium phosphate nanoparticles. The particles are aged for 36 h at room temperature. A solution of dexamethasone in ethanol was added to calcium nitrate tetrahydrate solution that was further added to ammonium phosphate dibasic solution and stirred for 30 min at 55 °C, aged for 36 h at room temperature, to yield dexamethasone-loaded biphasic calcium phosphate nanoparticles (Chen et al. 2017b)

Chitosan	HTCC was dissolved in distilled water, and aqueous solution of sodium tripolyphosphate was added to it while stirring at room temperature, yielding aggregates, solution and opalescent suspension. HTCC/PTH-related peptide 1–34 (PTHrP1–34) nanoparticles were formed spontaneously upon incorporation of the sodium tripolyphosphate solution and HTCC solution containing PTHrP1–34, with the conditions of stirring at room temperature for 2 h. The precipitates separated from suspension were dried by a freeze-dryer (Zhao et al. 2010)
	Iron oxide nanoparticles were prepared in an aqueous medium by addition of ferric chloride to ferrous sulphate solution, followed by constant stirring for 20 min under a nitrogen atmosphere at 45 °C to remove any dissolved oxygen. Ammonia was then added, and the mixture was stirred constantly for 30 min until a precipitate was formed. This precipitate was washed with deionised water to remove excess ammonia, leaving a brown precipitate. The solution was mixed continuously and chitosan was added. The pH was adjusted to 4 using phosphoric acid. The solution was stirred constantly for 12 h. The nanoparticles were then separated using a permanent magnet and washed with deionised water. The precipitate was directly dried in a vacuum oven at 50 °C for 20 h (Zhao and Guo 2016)
	Chitosan solution was prepared in acetic acid and PTH 1–34 was added to it, followed by incubation at 4 °C for 4 h under constant stirring. To this mixture sodium tripolyphosphate was added dropwise to generate a nanosuspension. PEG200 was added to the polymer and stirred for 1 h. The suspension obtained thereafter was centrifuged, and the pelleted particles were resuspended in deionised water (Narayanan et al. 2013)
	Chitosan was dissolved in acetic acid and mixed with varying amounts of rhBMP2. 2-N,6-O-sulphated chitosan (2,6SCS) in sodium sulphate was prepared as the negatively charged polyanion formulation. rhBMP2/NPs were formed by adding an equal volume of rhBMP2/chitosan solution into 2,6SCS/sodium sulphate solution, drop by drop, and vortexing at maximum speed for 60 sec at room temperature. The precipitated particles were lyophilised until required (Cao et al. 2014)
	Ionic gelation process; tripolyphosphate (TPP) aqueous solution was added to chitosan solution and stirred at room temperature for 30 min. The ionic interaction between positively charged amino groups of chitosan (dissolved in acetic acid) and negatively charged TPP resulted in formation of nanoparticles (Saini et al. 2015)
	Chitosan was dissolved in acetic acid, and minocycline was added to the chitosan solution with constant stirring. Subsequently, TPP in distilled water was added dropwise into the chitosan minocycline mixture under vigorous magnetic stirring. The resulting mixture was stirred for another 20 min and then centrifuged. Subsequently, the precipitate was suspended in distilled water and centrifuged again and freeze-dried (Ma et al. 2016)
	Chitosan was dissolved in acetic acid. After adding polyanionic TPP to chitosan solution and adjusting the final pH to 5.4, chitosan nanoparticles were formed under magnetic stirring for 30 min. Then nanoparticle suspension was sonicated for 5 min for better dispersion and left for 15 min at room temperature (Li et al. 2016a)
	Chitosan/TPP/hyaluronic acid nanoparticles (CTH NPs) were prepared using the ionotropic gelation technique with some modification. Chitosan was dissolved in acetic acid solution, and the pH was adjusted to 5.5 by adding NaOH. TPP and hyaluronic acid were dissolved in deionised water (pH 5.5) and filtered. Nanoparticles were formed instantaneously upon the dropwise addition of TPP/hyaluronic acid solution to chitosan solution under magnetic stirring. CTH/antimiR-138 NPs were prepared by incorporating the antimiR-138 in the hyaluronic acid/TPP phase and then added to the chitosan solution to form CTH/antimiR-138 NPs (Wu et al. 2016)

(continued)

Table 10.1 (continued)

Nanoparticle	Method of synthesis
	Chitosan/chondroitin sulphate nanoparticles were prepared based on ionic gelation of chitosan cations with chondroitin sulphate anions with minor modifications. The colloidal suspension of chitosan/chondroitin sulphate nanoparticles were obtained through the electrostatic interaction between a solution of chitosan at pH 2, 3 and 4 and chondroitin sulphate. The chitosan solution was slowly added to the chondroitin sulphate solution, and the mixture was homogenised by a magnetic stirrer. The resulting suspension was ultrasonicated using a probe sonicator by probe TT13 in amplitude 40% to form chitosan/chondroitin sulphate nanoparticles. Rosuvastatin-loaded nanoparticles were prepared by adding a constant amount of drug to the chondroitin sulphate solution prior to the interaction with the chitosan solution. The resulting nanoparticle suspension was centrifuged for 10 min at 25 °C and lyophilised to obtain the white powder of rosuvastatin-loaded chitosan/chondroitin sulphate nanoparticles using freeze-dryer (Rezazadeh et al. 2018)
Gelatin	Glutaraldehyde was used to cross-link gelatin nanoparticles with a molar ratio of 2 (gluteraldehyde/amine group, NH_2). After cross-linking at room temperature for 16 h, glycine solution was added to the gelatin nanoparticles suspension to block the unreacted aldehyde groups. After three cycles of centrifugation and resuspension in Milli-Q water by vortexing, the pH of the suspension was adjusted to 7 (Farbod et al. 2016)
	BSA solution was added to NaCl solution under constant stirring at room temperature. The mixing was allowed to proceed for 15 min, and BMP2 solution (in ddH_2O) was added into this solution. This aqueous phase was then desolvated with dropwise addition of ethanol after 2 h of incubation. The mixture was stirred under room temperature for 3 h and the nanoparticles were coated with PEG-modified PEI in NaCl solution. The coating was allowed to proceed for 1 h on an orbital shaker, and the coated nanoparticles were extensively dialysed (Zhang et al. 2010)
	Modified two-step desolvation method; gelatin was dissolved in distilled water and was heated gently to 50 °C. pH of the solution was adjusted to 3.6–3.7 using HCl followed by rapid addition of acetone to the solution that leads to formation of white precipitate. The precipitate was collected and redissolved in deionised water, and the pH was maintained at 3.6–3.7 using HCl. Then the solution was constantly stirred at 40 °C, and acetone was added at a constant speed yielding a milky white solution. The nanoparticles formed were cross-linked by adding glutaraldehyde and were stirred continuously for 1 h followed by overnight incubation at room temperature without stirring. After incubation, the nanoparticle suspension was neutralised by adjusting the pH to 7.4 using NaOH. The neutralised nanoparticle suspension was pelleted and resuspended in methanol (Binulal et al. 2012)
	Dextran sulphate solution was prepared in distilled water, and BSA powder was dissolved in it. Chitosan solution was prepared in acetic acid. Both these solutions were irradiated with UV for 30 min. Dextran sulphate-BSA solution was added to chitosan solution under vigorous stirring in sterile environment, which results in formation of dextran sulphate chitosan nanoparticles instantaneously. These nanoparticles are recovered by centrifugation at room temperature (Valente et al. 2013)
Protein	Calcium nitrate and ammonium phosphate solutions were sequentially added to PEGylated hyaluronic acid solution with constant stirring. During the procedure it was made sure that the molar ratio of Ca^{2+} and COO^- remains fixed at 1:2, so as to prevent homogeneous growth of calcium phosphate minerals (Alam et al. 2017)

Polysaccharide	TNF-α siRNA solution or siRNA solution in TE buffer was added to DOTAP/PLGA solution in chloroform. The mixture was sonicated for 90 s to obtain a water-in-oil emulsion. PVA in water was added to the emulsion, which was sonicated for another 1 min, resulting in a water-in-oil-in-water double emulsion. The double emulsion was subsequently diluted with PVA in water and left under agitation overnight to allow for evaporation of residual chloroform. For isolation of the nanoparticles, the dispersion was centrifuged for 12 min at 4 °C. The supernatant was discarded, and the pellet containing the nanoparticles was re-dispersed in DEPC-treated water (Te Boekhorst et al. 2012)
	Simvastatin (SIM) and hydrogenated soya phosphatidylcholine (S100) were dissolved in methylene chloride and conjugated by stirring for 30 min. The solution was then evaporated under vacuum at room temperature. After methylene chloride was evaporated, the final SIM-S100 drug compound was obtained. PLGA NPs and tetracycline (TC)-PLGA NPs were prepared via the solvent emulsification method. Briefly, the SIM-S100 drug compound, octadecylamine (ODA)-FITC, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide (DiR), PLGA and TC-PLGA2000, or the PLGA2000, were dissolved in methylene chloride. The mixture was quickly dispersed into PVA solution under high shear and dispersed for 10 min, followed by homogenisation via a probe sonicator. The emulsion was then evaporated under vacuum until methylene chloride was evaporated. The PLGA NPs and TC-PLGA NPs were isolated by centrifugation and were washed with deionised water several times (Wang et al. 2015)
	The oppositely charged blank PLGA nanoparticles were prepared by a solvent diffusion method. PLGA was dissolved in acetone and then the solution was added into polyvinylamine (PVAm) or poly(ethylene-co-maleic acid) (PEMA) surfactant solution through a syringe pump under stirring. The solution was stirred overnight to evaporate acetone. Nanoparticles were collected by centrifugation. The nanoparticles were centrifuged and resuspended using deionised water to remove excess surfactant. A fine powder of nanoparticles was obtained by lyophilisation for ~2 days (Wang et al. 2010)
Poly(lactic-co-glycolic acid) (PLGA)	PTH (1–34)-loaded PLGA nanoparticles were prepared using a modified double emulsion-solvent diffusion method. Briefly, aqueous phase containing total protein (PTH and BSA were at ratios 1:9, 1:4 and 1:2.33, respectively), containing trehalose in PBS, was added to PLGA solution in ethyl acetate and probe sonicated for 2 min. The resulting emulsion was transferred into PVA (pH 8.5) solution and sonicated further for 2 min to form secondary emulsion. This mixture was transferred into PVA (pH 8.5) solution and homogenised for 3 min to stabilise the double emulsion and facilitate the solvent diffusion. The organic solvent was evaporated by stirring the double emulsion with normal saline at 30 °C for 3–4 h (until the solvent was evaporated). The nanoparticles were collected by ultracentrifugation for 30 min, washed with purified water and freeze-dried (Gentile et al. 2015)
	PLGA nanoparticles were prepared using a modified double emulsion-solvent diffusion method. 1 ml of BSA aqueous phase, containing trehalose in PBS, was added to PLGA solution in ethyl acetate and subjected to probe sonication for 2 min. The resulting emulsion was transferred into PVA (pH 4.5) solution and sonicated for 2 min. After 2 min of sonication, the mixture was transferred into PVA solution and homogenised for 3 min to form a double emulsion. The organic solvent was evaporated by stirring the double emulsion with normal saline at 30 °C for 3–4 h. The nanoparticles were collected by ultracentrifugation, washed with purified water and freeze-dried (Nandagiri et al. 2011)
	Emulsion method; a PLGA solution in dichloromethane was added dropwise to a PVA solution in deionised water. This emulsion was sonicated and then stirred overnight at room temperature to allow solvent evaporation and particle formation. The resulting particle suspension was centrifuged, and the pellet suspension in deionised water was lyophilised to obtain PLGA nanoparticles. To modify the PLGA particles with NHS, PLGA nanoparticle suspension in MES buffer (pH 4.75) was mixed with EDC for 30 min at room temperature. NHS was then added to the solution and allowed to mix for 2 h at room temperature to graft NHS onto the surface of PLGA nanoparticles. The NHS-modified PLGA nanoparticles (PLGA-NHS) were purified by centrifugation, then subsequently washed with deionised water and lyophilised (Pandey et al. 2018)

10.2 Inorganic Nanoparticles

10.2.1 Metal Oxides

10.2.1.1 Iron (Fe)

It has been proved that the bone tissue has the ability to recognise the mechano-electrical conversion (Singh et al. 2014). Additionally, it has also been shown that the fate of stem cells could be altered to osteogenic or adipogenic by modifying the nano-topography (nano-grooves, nano-protrusions or nanofibrous) for focal adhesion formation, cytoskeleton organisation and cellular expansions. This nanoscale mechanical stress-mediated cellular force isotropy has been shown to drive osteogenic differentiation of MSCs by activation of focal adhesion kinases (FAK) and runt-related transcription factor 2 (RUNX2) (Wang et al. 2016a). Therefore, fabrication of functionalised magnetic nanoparticles as carriers for bioagents has revolutionised the conventional concept of scaffold-based tissue engineering. These magnetic carriers release the growth factors (that they are transporting) that are taken up by the tissues during regeneration process (Bock et al. 2010).

Among the various forms of iron oxides, maghemite (γFe_2O_3) and hematite (αFe_2O_3) find numerous applications as contrast agents in magnetic resonance imaging (MRI), magnetically guided drug or gene delivery, cell targeting, cancer therapy and photo-assisted electrolysis of water, respectively. Incorporation of γFe_2O_3 nanoparticle (γ iron oxide nanoparticle—γIONPs) and αFe_2O_3 nanoparticles (αIONPs) into calcium phosphate cements (CPC) has been reported to improve the injectability and compressive strength of these cements, without adversely affecting the physico-chemical setting reactions and cytocompatibility. This incorporation changes the surface topography and increases the surface area for cellular adhesions. The release of incorporated IONPs and their internalisation through endocytosis induces osteogenic differentiation via MAPK pathway (Wang et al. 2016a). Additionally, the lysosomal transfer of free iron ions and their release into the cytoplasm enhances the cell growth (Xia et al. 2018a). Similar osteoconductive properties are demonstrated by the superparamagnetic responsive nanofibrous scaffolds prepared from a mixture of PLA, nHAp and superparamagnetic γFe_2O_3 nanoparticles, under static magnetic field (Meng et al. 2013). Static magnetic fields magnetise the superparamagnetic scaffolds to generate mechanical forces, which in turn inhibit toll-like receptor (TLR) 2/4 activation and direct the macrophages in the vicinity towards a M2-like phenotype. This results in enhancement of the expression of growth factors such as vascular endothelial growth factors (VEGF), transforming growth factor (TGF) β and platelet-derived growth factor (PDGF); inhibition in secretion of pro-inflammatory cytokines such as interleukin (IL) 1β, IL6, tumour necrosis factor (TNF) α, interferon (IFN) γ and membrane cofactor protein (MCP) 1; and also inhibition of cytokines associated with osteoclast differentiation such as matrix metalloproteinase (MMP) 9 and tartrate-resistant acid phosphatase (TRAP). This orchestrates the transition of guided macrophages from an inflammatory to regenerative phenotype (Hao et al. 2017). Also, under an external magnetic field, a

superparamagnetic responsive scaffold degrades faster, and a magnetic stimulation recruits more macrophages to the scaffold (Meng et al. 2013). Magnetic nanoparticles do not exhibit any magnetisation after removing the external magnetic field (Li et al. 2016c). Thus, a weak magnetic force stimulation significantly promotes bone regeneration and repair (Meng et al. 2013).

Toxicity of metal oxide nanoparticles depends majorly on the size, type, contact angle and zeta potential (Suriyaprabha et al. 2015). The degree of atomic order or crystallinity in the iron oxide lattice and the dispersity in terms of size and shape of the nanoparticle affect their performance in biomedical application. The size of the magnetic particles significantly influences their behaviour in magnetic field. A smaller size (~10–15 nm, smaller than the exchange interaction critical size) exhibits superparamagnetic property at certain temperature ranges (Li et al. 2016c). For example, Al_2O_3 nanoparticles with a particle size of 50 nm show higher cell death as compared to even a higher concentration of particles with a size of 40 nm. Thus, the particle size plays an important role in governing the antioxidant activity and biocompatibility (Suriyaprabha et al. 2015).

Pure magnetic particles are toxic and lack functional groups or surface-coating materials, which limits their biomedical applications. Therefore, surface modification approaches by physical adsorption of polymer layers or by chemical conjugation improve their applicability in tissue engineering (Huang et al. 2011). Generally, magnetic nanoparticles are divided into metal oxides (usually Fe_3O_4 or Fe_2O_3; ferrite, $BaFe_{12}O_{19}$, $CoFe_2O_4$), pure metals and magnetic nanocomposites (Li et al. 2016c). Coating materials with good biocompatibility stabilise these magnetic nanoparticles in physiologic fluids and provide chemical functionality for additional modification. Organic coatings such as glycosaminoglycan, carboxymethylated dextran, polyethylene glycol (PEG), polyvinyl alcohol (PVA), poloxamers, polyoxamines and polylactic acid (PLA) and functionalisation with inorganic materials such as silica and gold are the commonly used methods for surface modification (Huang et al. 2011). It has been found that citrate-functionalised magnetic nanoparticles display reduced agglomeration (Wang et al. 2016a). Encapsulation of magnetic nanoparticles and quantum dots into a silica shell by reverse microemulsion; fine-tuning of the relaxometry of Fe_2O_3/SiO_2 core shell nanoparticles by thickness adjustment of coated silica layer; coating of hollow magnetic spheres with thin layer of SiO_2 by aerosol pyrolysis; and fabrication of magnetic gold nanoshells by embedding gold nanoshells within Fe_3O_4 nanoparticles are few of the approaches to functionalise the magnetic nanoparticles (Huang et al. 2011).

Magnetic nanoparticles coated with RGD (Arg-Gly-Asp) peptide have been used for magnetisation of HA/collagen composite scaffolds to enhance osteogenesis (Bock et al. 2010). Two more approaches for magnetisation of HA/collagen (70/30 wt%) scaffolds have been developed: (1) direct nucleation of biomimetic phase and superparamagnetic nanoparticles on self-assembling collagen fibres (MAG-A) and (2) scaffold impregnation in ferrofluid solution (MAG-B). Both the magnetised scaffolds displayed variation in cellular responses, which was also governed by the site of implantation. The diaphyseal implantation was associated with thin trabeculae in MAG-A, whereas a thicker trabecula in MAG-B. The epiphyseal

implantation of MAG-A scaffold was observed to undergo extensive resorption and replacement by woven trabecular bone, whereas MAG-B scaffold showed more residual Fe. However, both the groups displayed presence of Fe-laden macrophages in the periphery of the implant and were related to reparative tissue (Panseri et al. 2012). Different percentages of magnetite nanoparticles have also been incorporated into silk fibroin/chitosan scaffolds to enhance the repair and regeneration process (Aliramaji et al. 2017). The magnetite nanoparticles have been used for fabrication of magnetite cationic liposomes, to develop a novel magnetic force-based tissue engineering approach (Ho et al. 2007).

In order to fabricate poly(L-lactic) acid (PLLA)/Fe_3O_4 nanofibrous scaffold, Fe_3O_4 nanoparticles were co-precipitated in the presence of PLLA. As PLLA is insoluble in water, its dissolution requires selection of a strong polar solvent like trifluoroethanol (TFE), and therefore Fe(II) chloride tetrahydrate, Fe(III) chloride hexahydrate and sodium hydroxide were dissolved in TFE to yield Fe_3O_4 nanoparticles. After incorporation of PLLA into the system, PLLA/Fe_3O_4 nanoparticles were precipitated out using deionised/deoxygenated water, because oxygenation of Fe_3O_4 nanoparticles causes loss of their paramagnetism. PLLA/Fe_3O_4 nanofibrous scaffolds have been shown to enhance cell proliferation and maintain cellular morphology of preosteoblast cell lines (Shan et al. 2013). Polycaprolactone (PCL)scaffolds incorporated with magnetic nanoparticles have been shown to affect the physico-chemical, mechanical and biological properties during bone regeneration processes (Singh et al. 2014). Chitosan-coated IONPs have been shown to be cytocompatible and also enhanced osteoblast activity (Zhao and Guo 2016). Incorporation of magnetic nanoparticles has been found to improve the hydrophilicity of nanofibres (Wang et al. 2016a). Forsterite nanopowder formulated by sol-gel process has been observed to exhibit a good bioactivity and biocompatibility with a potential to be used as bone repair material (Li et al. 2016c).

10.2.1.2 Magnesium (Mg)

Magnesium is a biocompatible, biodegradable, low cost and environment-friendly material that exists naturally in the human body. Magnesium is involved in basic cellular functions such as transportation of K^+ and Ca^{2+}, modulation of signal transduction, energy metabolism and cell proliferation (Roh et al. 2017). Mg^{2+} ions are found to be highest in concentration in the bone (approx. 1% of bone ash) and reside along the edges of bone HA, contributing to the mineral density and mechanical properties of the bone. Divalent Mg^{2+} (as well as Ca^{2+}) ions have been associated with bone regeneration through activation of alkaline phosphatase (ALP) and integrins for ligand binding (thus facilitating cellular attachment, proliferation and migration). Unmodified bulk magnesium under physiological conditions degrades and releases Mg^{2+} ions, hydroxide (OH^-) ions and hydrogen (H_2) gas in the surrounding fluid, whereas nanostructured bulk magnesium supports cellular adhesion (Hickey et al. 2015).

Bisphosphonates have been reported to exhibit excellent binding affinity to multivalent cations such as Mg^{2+}. Based on this observation, mixing of methacrylated hyaluronic acid, acrylated bisphosphonate and magnesium chloride solutions has been demonstrated to cause formation of cross-linkable acrylate via efficient chelation between acrylated bisphosphonate and Mg^{2+}. These nanoparticles bearing acrylate group on the surface not only act as multivalent cross-linker to strengthen the hydrogel network but also promote mineralisation of hydrogel, with sustained release of Mg^{2+} (Zhang et al. 2017b). Magnesium oxide (MgO) nanoparticles have also demonstrated their potential as bioactive fillers in various matrices/polymer composites (e.g. PLLA matrices), for biomedical scaffold applications (Suryavanshi et al. 2017). It has been reported that incorporation of MgO nanoparticles reduced the harmful exothermic reactions of poly(methyl methacrylate) (PMMA) during solidification and also increased radiopacity, thus enhancing the toughness of bone cement interphases (Khandaker et al. 2013). PCL/HA/MgO scaffolds treated with O_2 and N_2 plasma have been shown to display early-stage differentiation of MC3T3-E1 cells (preosteoblast cells) (Roh et al. 2017). Surface treatments of scaffolds using O_2 and N_2 plasma change the key physico-chemical surface properties by introduction of amine, imine, amide, nitrile, carboxyl and other functional groups onto the polymer surface (Amiri et al. 2016).

10.2.1.3 Zinc (Zn)

An ideal approach for bone tissue engineering requires tissue integration prior to bacterial adhesion. Majority of implant rejections and/or biomaterial-associated infections in orthopaedics are caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*, that are associated with formation of biofilms, septic arthritis and osteomyelitis (Ribeiro et al. 2017). A prolonged use of antibiotics has several side effects and leads to development of antibiotic-resistant bacterial strains. The resistance mechanism involves microbial efflux system (that pumps out antimicrobial agents) or synthesis of exo-polymers (that prevents penetration of antimicrobial agents). Therefore, in order to achieve efficient bone tissue regeneration, the bone or dental implants should be coated or impregnated with metal-based antimicrobial particles to control biofilm formation (Abdulkareem et al. 2015; Ribeiro et al. 2017). Dehydrated bone is a piezoelectric material, but hydrated bone may not show piezoelectricity at normal physiological conditions. Piezoelectricity acts as a biological electric stimulation which can stimulate various biochemical reactions in cells, and energy conversion can be achieved in the process. Piezo-excited scaffolds possess strong potential for numerous antibacterial orthopaedic applications, and therefore the use of biocompatible piezoelectric materials such as zinc oxide, barium titanate and polyvinylidene fluoride (PVDF) has gained popularity as orthopaedic scaffold materials (Li et al. 2018). These metallic nanoparticles serve as an excellent antimicrobial agent due to their large surface area to volume ratio. Moreover, the use of multiple nanoparticles in a composite also provides a better synergistic

antimicrobial effect against opportunistic pathogens (Abdulkareem et al. 2015; Ribeiro et al. 2017).

Zinc nanoparticles have been shown to possess antibacterial activity, low toxicity, chemical stability, long-lasting action period and thermal resistance. Zinc has also been found to enhance osteogenesis and inhibit osteoclastogenesis probably through zinc trafficking, which involves zinc storage proteins and zinc transporters (Tripathi et al. 2012). Willemite (Zn_2SiO_4, zinc bioceramic containing silicate nanoparticles) has a similar chemical structure to the native bone minerals and thus possesses osteoconductive properties. NH_3 plasma-treated polyethersulphone (PES)-PEG electrospun fibrous scaffolds and bioactive glass-coated poly(lactic-co-glycolic acid) (PLGA) nanofibres, both loaded with Zn_2SiO_4 bioceramic nanoparticles (Zn_2SiO_4-PES-PEG), have been investigated to enhance osteogenic differentiation of human MSCs (Adegani et al. 2014; Amiri et al. 2016). Recently, there have been reports about fabrication of nanocomposite using chitosan, HA, zinc oxide (ZnO) and organically modified montmorillonite clay (OMMT, layered aluminosilicate), to improve mechanical properties, thermal stability and biocompatibility (Bhowmick et al. 2018). Incorporation of copper and zinc nanoparticles in chitosan/nHAp scaffolds has been found to significantly increase the swelling, protein adsorption and antibacterial activity, but decrease degradation (Bhowmick et al. 2018). At the same time, implant coating with ZnO nanoparticles has been shown to promote bone growth (Abdulkareem et al. 2015). ZnO nanoparticles in combination with HA nanoparticles are able to promote antimicrobial activity as well as provide a suitable substrate for growth, adherence and metabolic activity of osteoblasts (Memarzadeh et al. 2015).

Immobilisation of ZnO nanoparticles on carboxylated graphene oxide (GO-COOH) sheets has been demonstrated to provide antimicrobial and osteoinductive properties to the nanocomposite (Chen et al. 2016). Graphene is a flat monolayer of carbon atoms tightly packed into a 2D honeycomb lattice and is a basic building block for graphitic carbon materials of all other geometric arrangements with unique physical, chemical and mechanical properties (Raucci et al. 2017). Graphene is an allotrope of carbon and possesses extraordinary mechanical and electrical properties. It is widely used as surface modification coating or dopant in scaffolds, to enhance biocompatibility and promote osteogenic differentiation of stem cells. The chemical functionalisation of graphene with amino, hydroxyl or carboxyl groups on basal plane and over the edges enhances its osteoinductive properties (Chen et al. 2016).

10.2.1.4 Silicon (Si)

It has been demonstrated that silica (SiO_2) induces apatite formation, by chelating and providing sufficient atomic distance required by the crystal structure of bone apatite. Silica particles have a negative surface charge under physiological pH, and its silanol group enhances the stability of their suspensions in aqueous medium by making the surface lyophilic. Hydrated silica formed at the surface of glass

ceramics provides favourable sites for apatite nucleation, due to chemical bonding between Si-OH and apatite. The negatively charged Si-OH group electrostatically interact with positively charged Ca^{2+} ions and form a Ca-rich positive thin layer. This layer effectively attracts the negatively charged PO_4^{3-} ions to create amorphous calcium phosphate that eventually transforms into apatite (Lewandowska-Łańcucka et al. 2015).

Mesoporous silica nanoparticles have been extensively researched in biomedical applications, due to their large pore volumes, controllable particle size, accessible surface functionalisation, high-specific surface areas and biocompatibility. A mesoporous material is a structure that has an intermediate pore size between 2 and 50 nm and therefore acts as carrier for loading biomolecules and manages their release (Huang et al. 2017). Mesoporous silica nanoparticulate construct containing covalently bound bone morphogenetic protein (BMP) 2 peptide via aminosilane and loaded with dexamethasone has been suggested as a potential osteogenic delivery system (Zhou et al. 2015). Mesoporous silica nanoparticles have also been developed as an effective gene delivery tool for bone regeneration. Green fluorescent protein (GFP)-tagged BMP2 plasmid DNA (pDNA)-loaded amine-functionalised mesoporous silica nanoparticles have been demonstrated to promote osteogenesis in MSCs upon transfection (Kim et al. 2013). Along with this, the mesoporous silica nanoparticles have also been used for local delivery of fibroblast growth factor (FGF) 2 and BMP7-derived bone-forming peptide (BFP) and have been shown to enhance osteogenic differentiation (Luo et al. 2015; Huang et al. 2017).

The copper-mesoporous silica nanoparticles, when phagocytosed by the immune cells, have been found to initiate pro-inflammatory cytokines release, induce osteogenic (by activation of oncostatin M pathway in MSCs) or angiogenic factors and suppress osteoclastogenic factors by the immune cells (Shi et al. 2016). Bioactive ions such as copper (Cu) and cobalt (Co) are reported to possess hypoxia-mimicking capacities and exert physiological effect at very low concentrations, as they are less sensitive to micro-environmental conditions such as pH and temperature. Cu-deficient animals have been reported to possess brittle bones probably due to reduced cross-linking of collagen. These bioactive ions endow the mesoporous silica nanoparticles with both osteogenic and angiogenic capacities and, therefore, eliminate the need of osteogenic/angiogenic cytokines such as BMP2 or VEGF. Cu ions not only impede the activity of exogenous prostaglandins but also inhibit lysosomal enzyme-mediated bone resorption (Shi et al. 2016).

There are reports that laponite (bioactive silicate nanoplatelets based on synthetic silicate) is uptaken by stem cells via clathrin-mediated endocytosis and induces osteogenic differentiation even in the absence of osteoinductive factors such as BMP2 or dexamethasone (Mihaila et al. 2014). Mesoporous calcium silicate nanoparticles have been shown to possess antibacterial activity (due to alkaline micro-environment created by release of Ca^{2+} ions). The Si-OH functional groups of calcium silicate-based materials act as nucleation centres for apatite precipitation, thus promoting bone tissue repair (Huang et al. 2017). Also, PCL/silica nanoparticle composite biomaterials, loaded with dexamethasone, have been shown to enhance stem cell differentiation towards osteogenic lineage (de Matos et al. 2013).

Bioactive Glass

Silica is also a well-known component of bioactive glass (BG) and stimulates apatite formation. The ability of silica nanoparticles to undergo in situ gelation under physiological temperature makes them a promising candidate for synthesis of bioactive injectable systems (Lewandowska-Łańcucka et al. 2015). Fabricated BG nanoparticles demonstrate a negative zeta potential in distilled water, which facilitates attachment and proliferation of bone cells. Ionic dissolution products of BGs have been found to induce osteogenic differentiation of stem cells, by acting as gene activation materials (Luz et al. 2012; Tavakolizadeh et al. 2017).

Uncontrolled and excessive ionic release from BG nanoparticles causes change in pH of the local environment, which is lethal to the cells. In addition, the biological response due to nanosized particles is different from the one obtained from larger particles of same chemical composition. Microcontact printing has been demonstrated to provide precise control of nanoparticle density at the surface, thereby controlling ionic release or inefficient particle concentration (Luz et al. 2012).

Inclusion of BG nanoparticles in carbon nanofibres and PES nanofibres has significant effects on the bone regenerative process (Ardeshirylajimi et al. 2015; Cheng et al. 2017). SiO_2-CaO-P_2O_5 has been employed as bone filling material in dental application, as bioactive coatings or as 3D scaffold (Tavakolizadeh et al. 2017). BG nanoparticles were used to form spherical aggregates on biomimetic superhydrophobic surfaces that incorporated SiO_2-CaO sol-gel-based nanoparticles (Luz and Mano 2012). Incorporation of BG nanoparticles in electrospun PLLA scaffold has been shown to improve its osteoconductivity (Mahdavi et al. 2017).

10.2.2 Metals

10.2.2.1 Gold (Au)

The osteogenic response generated by gold nanoparticles (AuNPs) is highly sensitive to particle size. 20 nm gold nanoparticles were reported to be taken up by osteoblast (40 nm AuNPs do not show osteogenesis), where they accumulate in perinuclear compartments and vesicular structures close to the nucleus (Zhang et al. 2014). Some of the studies also report greater cellular uptake of AuNPs ranging in size from 30 to 50 nm (Ko et al. 2015). Few of the previous research show bioaccumulation of AuNPs in various internal organs on the basis of their size (5 nm, heart and kidney; 10 nm, liver; 30 nm, spleen) (Lee et al. 2016a). AuNPs are usually surface functionalised with L-glutamic acid, 2-aminoethylphosphonic acid or alendronate, which provides it a primary amine for binding to carboxylate, phosphonate or bisphosphonate groups, respectively, and targets calcium in the bone. Functionalised AuNPs exhibit relatively higher water solubility and low viscosity as compared to iodinated molecular contrast agents (Ross and Roeder 2011).

AuNPs have been shown to promote osteogenic differentiation and inhibit adipogenic differentiation of mouse MSCs through p38 MAPK pathway. AuNPs weaken the production of reactive oxygen species (ROS) and increase the expression of glutathione peroxidase 1, thereby inhibiting the receptor activator of NFκB ligand (RANKL)-mediated osteoclast differentiation of bone marrow macrophages. Conjugation of AuNPs with alendronate has been shown to inhibit the expression of osteoclast-specific genes such as TRAP, osteoclast-associated receptor (OSCAR), c-Fos and nuclear factor for activated T-cells (NFATc)1 (Lee et al. 2016a; Xia et al. 2018b).

Bone marrow MSCs of type 2 diabetic rats have been identified to express strikingly higher levels of microRNA (miR204). Inhibition of miR204 has been found to significantly increase the osteogenic capacity. Based on this background, the osseointegration in diabetic rat models using titanium implants employed coating with miR204 inhibitor conjugated to AuNPs (AuNP-antagomiR204), followed by their dispersal in PLGA solution (Liu et al. 2017). Similar to miR204, miR138 also serves as a negative regulator of osteogenesis through inhibition of FAK-ERK1/2 signalling pathway. Another microRNA, miR29b, has been shown to positively regulate osteogenesis via Wnt and MAPK pathway. Polyethyleneimine (PEI)-capped AuNPs have been found to efficiently transduce miR29b into human MSCs via compatible ER stress and induce osteogenic differentiation (Pan et al. 2016). Incorporation of carboxylated AuNPs into polystyrene scaffolds has been shown to promote formation of HA (Terranova et al. 2017). Loading of AuNPs into chitosan/pectin composites also promotes osteoblast proliferation and growth (Tentor et al. 2017). Conjugation of 28 nm AuNPs on the surface of silanised titanium implants via Au-S bonds showed remarkable increase in the expression of collagen type I, RUNX2, osteocalcin, bone sialoprotein and higher levels of ALP activity (Heo et al. 2016).

Incorporation of AuNPs into CPCs has been found to improve the properties of CPCs such as finer microstructure, improved wetting and protein adsorption as well as cellular attachment and spreading. This also enhanced the osteogenic differentiation of dental pulp stem cells by increasing the ALP activity, osteogenic gene expression and bone matrix mineral synthesis. Few of the reports also emphasise that AuNPs generate mechanical stress on the cells due to their endocytosis and eventually regulate the Yes-associated protein (YAP) activity (Xia et al. 2018b). Impregnation of AuNPs into hydrogels enhances the mechanical properties of hydrogel and at the same time serves the purpose of systemic delivery of biomolecules (Heo et al. 2017a).

10.2.2.2 Silver (Ag)

Silver possesses antibacterial activity, as silver nanoparticles (AgNPs) penetrate inside the bacteria and damage the phosphorous- and sulphur-containing compounds like DNA. Oxidised form of silver (Ag^+) nanoparticles has been found to inhibit the enzymatic activities, prevent DNA replication and disrupt the bacterial

cell membrane (Ribeiro et al. 2017; Hasan et al. 2018). Hence, they are used to reduce biofilm formation on the implant surfaces. AgNPs via tissue fluid-mediated release of metallic silver particles or Ag^{+} ions have been shown to be involved in generation of ROS, apoptosis and replacement of ions (such as Ca^{2+} and Mg^{2+}) that are essential for cellular functions (Geng et al. 2017). Biological toxicity of silver gets reduced upon biotransformation of silver into silver sulphide (through complex interactions with serum proteins), a more stable and less toxic compound (Hasan et al. 2018). This could probably be the detoxification pathway during regeneration of bone tissue adjacent to the silver-coated implant surface (Geng et al. 2017).

AgNP-coated titanium implants have been shown to reduce biofilm formation and enhance in vivo bone formation and neovascularisation within the pores of the implants. The bone growth involves both distant (from defect to implant surface) and contact (from implant surface to defect) osteogenesis, leading to faster osseointegration (Geng et al. 2017). Fabrication of biocomposite scaffold containing chitosan/nano-HA/nano-silver particles (CS/nHAp/nAg) by freeze-drying technique was found to favour cell penetration, adhesion and spreading in addition to providing mechanical strength (Saravanan et al. 2011). Incorporation of AgNPs into bifunctional cellulose nanowhiskers is a good option for bone tissue engineering applications. Cellulose nanowhiskers are nanosized cellulose fibres synthesised by acid hydrolysis of cellulose (Hasan et al. 2018).

10.2.2.3 Calcium Phosphate (CaP)

Calcium is one of the most important elements of human health. Our diet provides good amount of calcium, but its absorption by the body depends on the amount of soluble calcium in the duodenum and proximal jejunum. Calcium deficiency in children and postmenopausal women has been associated with low bone density and is one of the major causes of disorders such as osteoporosis, loosening of prosthesis after arthroplasty and increased risk of fractures. Supplementation of calcium in the form of calcium carbonate, calcium hydroxyapatite, calcium lactate and calcium gluconate has been the common approach for improving calcium deficiency (Guo et al. 2015; Zhao and Guo 2016). Calcium phosphate (CaP) implants have been used as tissue engineering scaffolds for delivery of drug and provide mechanical strength to the regenerating site. Initial burst and uncontrolled release of drug from implant surface are not effective as far as local delivery of drug is concerned. Adsorption of drug and its release from CaPs primarily depend on chemical and electrostatic interactions between CaP and drug molecule (Tarafder and Bose 2014). Ca^{2+} ions enhance the anti-osteoclastic effect of nitrogen-containing bisphosphonates by enhancing their endocytic internalisation in vitro. CaP particles act as nucleation sites for promoting HA precipitation (Kettenberger et al. 2017). One of the study indicated that PLA scaffolds created with single component and porous fibre scaffolds exhibit an initial burst release of tricalcium phosphate nanoparticles, whereas core-sheath fibres show a steady release (Asli et al. 2012). Other than the

bone regenerative activities, calcium oxide nanoparticles have also been demonstrated to possess antibacterial activity (Münchow et al. 2016).

Injectable bone cements exhibit self-setting and in situ hardening properties. The first CPC approved by the Food and Drug Administration (FDA) was based on tetracalcium phosphate and dicalcium phosphate anhydrous (Xia et al. 2018b). Injectable methyl-cellulose-CaP nanoparticle nanocomposites, synthesised by hydrogelation, are suitable for injection at body temperature due to their rapid sol-gel transition (Kim et al. 2018). Formulation of morphogenetically active bio-ink from amorphous microparticles of Ca^{2+}, which are derived from calcium nanoparticles, has successfully been utilised for fabrication of 3D-printed biomaterials (Neufurth et al. 2017).

Collagen peptide extracted from marine fish scales has been used for synthesis of collagen peptide-chelated calcium nanoparticles encapsulated in alginate (Guo et al. 2015). Collagen scaffolds coated with β-tricalcium phosphate nanoparticles loaded with FGF2 have been shown to promote cell-ingrowth behaviour and angiogenesis in subcutaneous tissues (Murakami et al. 2017). Amorphous CaP nanoparticles/β-tricalcium phosphate nanoparticles have also been found to be good candidates for inducing fast mineralisation, high biocompatibility and sustained drug release (Reddy et al. 2013; Fu et al. 2016; Lee et al. 2016b). Gelatin modified with CaP nanoparticles and PCL has been used to prepare a 3D-bilayer scaffold, with improved mechanical properties and enhanced bioactivity (Rajzer et al. 2014). Combination of gelatin and pectin for fabrication of scaffolds and their incorporation with biphasic CaP nanoparticles also enhance cell adhesion, viability and proliferation, leading to faster tissue regeneration (Nguyen et al. 2015). Incorporation of dexamethasone in biphasic CaP nanoparticles during nanoparticle synthesis locks dexamethasone in the biphasic CaP crystals for sustainable and simultaneous release (Chen et al. 2017b).

Yan et al. (2013) reported for the first time the synthesis of nanosized CaP particle in highly concentrated silk fibroin solution by in situ method. Fabrication of nanocomposites, containing tricalcium phosphate nanoparticles, nHAp, MgO nanoparticles and high-density polyethylene, has been reported to improve cell adhesion and ALP activity in osteoblast precursor cells (Pourdanesh et al. 2014). A similar approach using nano-biphasic CaP, PVA and platelet-rich fibrin has also been found to be highly effective for regeneration of critical-size segmental bone defects in rabbit (Song et al. 2018).

Bisphosphonates strongly bind to CaP surface because of chemical exchange of phosphate group, forming a layer of adsorbed bisphosphonate on the surface. The subsequent layers are formed by weak electrostatic interactions (Tarafder and Bose 2014). Bisphosphonates and its derivatives (such as alendronate, zolendronate and risedronate) act as potent osteoinductive molecule and therefore find wide application in treatment of skeletal disorders such as Paget's disease, osteoporosis and hypercalcemia (Kim et al. 2016).

10.2.2.4 Calcium Sulphate

Calcium sulphate is extensively used for bone grafting/regeneration purposes. After grafting in the body, it undergoes controlled degradation and breaks into calcium and sulphur ions. Calcium ions combine with phosphate ions to deposit calcium phosphate crystals (Mamidwar et al. 2006), which act as anchorage site for osteoblasts and eventually form the bone. Increased presence of growth factors such as BMP2, BMP7 and VEGF in defects grafted with calcium phosphate helps in formation of bone and blood vessel (Walsh et al. 2003). Additionally, it has barrier membrane properties that prevent ingrowth of soft tissue in the grafted defects. The only disadvantage associated with the use of calcium sulphate is its rapid degradation. To overcome this disadvantage, recently nanocrystalline calcium sulphate (nCS)-based materials have been developed. These materials have been found to undergo controlled degradation in vitro and in vivo. Moreover, various clinical studies have demonstrated highly efficient use of nCS in dental indications like extraction sockets (Kumari et al. 2014; Mazor et al. 2014), sinus augmentation cases (Mazor and Mamidwar 2013), furcation defects and intrabony defects (Kathuria et al. 2012).

10.2.2.5 Hydroxyapatite (HA)

Bone substitutes have been widely used to replace damaged bone because of their excellent mechanical properties. However, the mechanical mismatch between these substitutes and surrounding bone usually results in impairment of repair towards normal bone. The lack of tissue adherence and the accumulation of corroded metal ions (in case of metal implants) in the vicinity of the implant or at distant body parts (spleen, liver, draining lymph nodes) remain challenging for better functional recovery (Aydin et al. 2011; Ding et al. 2017). Development of injectable scaffolds reduces the level of damage to surrounding tissue, shortens the duration of surgery and accelerates the recovery of the damaged sites (Ding et al. 2017; Nazeer et al. 2017). However, HA coating is also one of the alternatives that passivates these metal implants and reduces their rate of corrosion in simulated body fluid medium (Rajapakse et al. 2016). Moreover, HA nanorods are also resistant to stress shielding effect and possess mechanical properties close to that of bone. These properties have been harnessed for fabrication of bone plates with high mechanical compressive strength (Aydin et al. 2011).

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is a biocompatible material that resembles the mineral component of bone and teeth (Saber-Samandari et al. 2018). Naturally the bone mineral content includes nanostructured non-stoichiometric nHAp (20 nm diameter and 50 nm length), substituted with ions of magnesium, fluoride and carbonate (Ingole et al. 2017). Biological apatite is a calcium-deficient HA crystal (Long et al. 2014) that is non-biodegradable and can only be removed or remodelled in the host. Upon implantation it dislocates within the tissue and therefore is usually used in combination with natural or synthetic polymers (Saber-Samandari et al. 2018). Typically, 20–40 nm-sized nHAp is desirable for assisting

cellular functions (Nazeer et al. 2017). HA has been reported to induce release of Ca^{2+} in the extracellular medium, through L-type calcium channel, that in turn triggers calcium/calmodulin (CaM)-dependent protein kinase pathway and also influences the fusion of osteoclast mononuclear precursors in bone (Xing et al. 2013; Maia et al. 2016). Biodistribution profile of nHAp shows a higher uptake in the liver and spleen (some uptake in the lungs), elimination through renal excretion and an increasing bone uptake over time, indicating a higher affinity to bone tissues (Maia et al. 2016).

Enhancement of the properties of HA majorly involves combination with other elements or materials and manipulation of microstructure (parameters such as morphology, crystallinity, grain and particle size, topography, porosity or compositional gradient) (Ignjatović et al. 2016). Popular methods such as sol-gel process at low temperatures and wet-chemical precipitation provide high yields of nHAp (Nazeer et al. 2017). nHAp has also been synthesised using eggshell waste as calcium source and ammonium dihydrogen phosphate as reactant (Ingole et al. 2017). Each of the three constitutive ions (calcium, phosphate and hydroxyl) of HA can be substituted with other ions that influence the physical properties and bioactivity of HA (Ignjatović et al. 2016). Several reports have revealed that substitution of HA with aliovalent mineral ions, such as Mg^{2+}, Sr^{2+} and Sm^{3+}, improves its bioactivity, biocompatibility, osteoconductivity and structural stability (Govindaraj et al. 2017). nHAp in polymeric matrices containing chitosan and/or gelatin exhibits strong chemical interactions via covalent bonding, ion-dipole interactions and complexation of Ca^{2+} ions with polymer amino, acetylamino and hydroxyl groups (Forero et al. 2017). Inclusion of non-ionic surfactants such as triton-X100 during preparation helps to produce colloidal HA nanorods with uniform and controlled size distribution (Rajapakse et al. 2016). nHAp is difficult to disperse and gets easily aggregated to form precipitates due to its inherent stable colloidal nature. But fabrication of bone tissue scaffolds requires colloidal stability of nHAp (Park et al. 2015). Pure formic acid has been reported to provide homogeneous dispersal of nHAp in chitosan solutions without agglomeration or sedimentation (Nazeer et al. 2017). The crystalline structure and morphology of nano-HA powders have also been found to be influenced by ultrasonication (Ingole et al. 2017).

Recently, the binary system of nanoparticle synthesis has gained popularity, as it involves addition of polymers such as chitosan, gelatin and alginate with HA. The ternary systems possess comparatively superior properties (in terms of homogeneous dispersal and thermal stability) and are synthesised by introducing components such as collagen, carbon nanotubes, PCL, carboxymethyl cellulose and montmorillonite. A ternary system of nanoparticle synthesis employing a co-precipitation approach for incorporation of nano-HA/β-cyclodextrin/chitosan has been found to yield a nanocomposite with enhanced antibacterial property and hemocompatibility. Cyclodextrins are water-soluble, cyclic oligosaccharides composed of α-D-glucopyranoside units obtained by enzymatic degradation of starch (Shakir et al. 2016).

BMP2/BMP2 peptide (20-mer synthetic peptide corresponding to residues 73–92, KIPKASSVPTELSAISTLYL) has been reported to be one of the most

potent protein in bone regeneration; however it has a very short half-life and is rapidly cleared from the local tissues. Its uncontrolled or off-site release may lead to heterotopic ossification, tumourigenesis and immune response. Formulation of delivery systems for controlled release of BMP2 at the defect site needs to be explored for achieving its regenerative effect (Mohammadi et al. 2018). Solvent casting/particulate leaching technique has been used to incorporate three biomolecules, cell adhesion-promoting $(K)_{16}$GRGDSPC peptides, osteoinductive nHAp and BMP2-derived P24 peptides into PLGA-[Asp-PEG]n scaffolds (Pan et al. 2014). Thiolated PLA scaffolds were treated with O_2 plasma to provide active moieties for recombinant human BMP2 (rhBMP2)-loaded liposomes and coated with nHAp (70 nm) (Mohammadi et al. 2018). Similarly, PCL/PLA/HA has been electrospun in different ratios to fabricate nanofibrous scaffolds, enhancing cellular attachment, proliferation and differentiation (Fang et al. 2010). Incorporation of nHAp into PEG hydrogel, containing the cell adhesion peptide RGD and MMP-sensitive cross links, has been shown to create a bone mimetic biodegradable scaffold for bone tissue engineering (Skaalure et al. 2018).

HA-based composites are brittle in nature and tend to crack under stress. Incorporation of 2,2,6,6-tetramethylpiperidine-1-oxyl(TEMPO)-oxidised bacterial cellulose (TOBC) nanofibres has been found to overcome these shortcomings of HA-based scaffolds and enhance the biocompatibility as well as load-bearing strength (Park et al. 2015). The use of air spray spinning with pressurised gas has also been successfully utilised for producing micronanofibres from plain PLA/HA nanocomposite in dichloromethane, ultimately reducing the brittleness of HA monoliths, improving the resistance to impact loading and repeated loading (Abdal-Hay et al. 2013).

PVA scaffolds containing bacterial ALP-synthesised carbonate nano-HA, doped with Zn^{2+} and Mg^{2+} (Ahmadzadeh et al. 2016) and PLA/carbonated calcium-deficient HA (CDHA) nanocomposites, have also been found to display osteoinductive properties (Zhou et al. 2011). Similarly, cellulose functionalised HA and cholecalciferol (vit D3)-loaded mesoporous silica nanoparticles have been shown to regulate extracellular levels of calcium and phosphorous (Sumathra et al. 2018). A biocomposite, fabricated using carbon fibre reinforced with nHAp/polyamide46, by extrusion technique was found to have similar modulus and better strength than natural bone (Deng et al. 2017). PLLA/collagen-type I/HA scaffolds were also demonstrated to have good biodegradability, cell proliferation and osteoconductive activity (Zhou et al. 2017). Poly(D,L)-lactic acid/nHAp scaffolds have been shown to induce rapid formation of bone-like apatite after soaking in simulated body fluid (Nga et al. 2015). HA/chitosan/carbon porous scaffolds (HCCPs) exhibit higher in vitro bioactivity and biocompatibility as compared to porous carbon fibre felts (PCFFs) (Long et al. 2014). But synthetic polyesters derived from lactides, glycolides and lactones have a disadvantage that their degradation products are acidic, which reduces the local pH, thus accelerating the degradation rate and inducing local and systemic inflammation. Three-dimensional bioprinted porous scaffolds

prepared by blending 1,6-hexanediol-L-phenylalanine-based poly(ester urea) (PEU) with HA nanocrystals, by fused deposition modelling, have been found to exhibit high elastic modulus without any evidence of local acidification-induced inflammatory response (due to the presence of acid-neutralising urea groups at each repeat unit). Fused deposition modelling involves layer-by-layer fabrication of a 3D structure that is able to adapt the outer edges of an implant to the patient-specific defect (Yu et al. 2017). 3D poly(butylene adipate-co-terephthalate) (PBAT, aliphatic aromatic co-polyester) scaffolds were incorporated with boron-doped nHAps, where nHAp not only induced osteoinductive properties but also minimised negative effects of acidic degradation products of the polyester on cellular functions (Arslan et al. 2018).

Injectable and degradable polysaccharide-based hydrogels were fabricated by Schiff-base linkages between oxidised alginate (containing aldehyde, –CH=O) and carboxymethyl chitosan (containing imine, –NH2 group). These hydrogels were further integrated with tetracycline hydrochloride-loaded calcium carbonate microspheres and nHAp, to enhance the mechanical and bioactive properties of the composite gel scaffold (Ren et al. 2018). Incorporation of nHAps with zoledronate was found to protect the regenerated tissue from degradation by antiresorptive effect of the bisphosphonate, thus resulting in larger mineralised regions (Kettenberger et al. 2017). Cellulose-graft-polyacrylamide/HA/titanium oxide (TiO_2) nanocomposite scaffolds with different amounts of TiO_2 were found to be promising candidates for bone tissue engineering (Saber-Samandari et al. 2018). Synthesis of mineral-substituted nHAp by using choline chloride-thiourea, a deep eutectic solvent, has been observed to provide a green way approach (Govindaraj et al. 2017).

Incorporation of nHAp into non-mulbury silk fibroin plays a major role in HA nucleation, by inducing β-sheet structure of silk fibroin, which results in exposure of hydrophilic groups such as –NH– and –COO–. These hydrophilic groups are responsible for adsorption of Ca^{2+} ions followed by electrostatic interaction of PO_4^{3-} and OH^-, leading to nucleation of HA and ultimately forming fibroin-nHAp (Behera et al. 2017). Injectable nanoscale systems, formulated by blending thixotropic silk nanofibre hydrogels and water-dispersible silk-nHAp, were found to imitate bone niche (Yu et al. 2017). The negative charge repulsion of silk shell has been found to inhibit the aggregation of nHAp, endowing them a homogeneous structure and excellent dispersibility in water (Huang et al. 2015). Gamma-irradiated HA-dispersed silk fibroin solution has been found to undergo chemical cross-linking to yield silk fibroin hydrogel (Kim et al. 2017).

Moreover, porous memory polymer of polyurethane/nHAp composite scaffold has potential applications in bone tissue engineering (Yu et al. 2018). Biomineralised HA nanocrystals-graphene has also gained popularity as bone implant material (Raucci et al. 2017). Additionally, HA nanocomposites containing risedronate and zinc have been demonstrated to be highly effective for treatment of postmenopausal osteoporosis in rat model (Khajuria et al. 2016).

10.3 Organic Nanoparticles

10.3.1 Lipid

Lipids are formulated into liposomes as nanocarriers for carrying drug molecules to the site of tissue engineering. For example, application of solid lipid nanoparticles for targeting lipophilic drugs such as chloroquine has been found to inhibit the production of TNFα and serve as promising tool for treatment of rheumatoid arthritis (RA) (Bhalekar et al. 2016). Similarly, solid lipid nanoparticles formulated using different ratios of vit D3, stearic acid, bees wax and sodium dodecyl sulphate (SDS) have been found to encapsulate variety of drug molecules where drugs are homogeneously dispersed within lipid matrix and loaded into the core of lipid shell or outside the lipid sphere (Demirbilek et al. 2017). A higher expression of hyaluronic receptor, CD44, on the surface of synovial lymphocytes, macrophages and fibroblasts in the inflamed joints of RA patients has been exploited for development of solid lipid nanoparticles encapsulating glucocorticoid prednisolone coated with hyaluronic acid (Zhou et al. 2018). By taking advantage of strong affinity of bisphosphonate to the bone surface, carboxyfluorescein-, doxorubicin- and lysozyme-loaded bisphosphonate liposomes were synthesised. These liposomes prolonged the in situ residence of drugs and also provided a sustained release platform for bone regeneration (Wang et al. 2011). Incorporation of bisphosphonate moieties, in cholesteyl-trisoxyethylenebisphosphonic acid (CH-TOE-BP), was shown to enhance the mineral affinity and retention time in the scaffold (Wang et al. 2012). Fabrication of lipophilic poly(3-hydroxybutyrate) nanoparticles loaded with hydrophilic BMP2 phospholipid complex has been found to successfully reduce the quick release of BMP2 and also enhance the osteogenic differentiation (Peng et al. 2016).

Simvastatin is a popular drug for treatment of hyperlipidemia and therefore has gained attention as osteoanabolic agent. In order to design a suitable delivery system, to negate off-target/side effects, a binary matrix nanomaterial was formulated using a blend of emulsifying wax and glyceryl monooleate. Glyceryl monooleate is an amphiphilic excipient and is used in preparation of stable drug-loaded oil-in-water nano-emulsion templates (Eskinazi-Budge et al. 2018). Another drug methotrexate (MTX) rapidly exits the joint cavity when administered intra-articularly, resulting in low drug concentration at the RA site. It also requires repeated joint needling, thus increasing the risk of infection. Development of MTX-loaded nanostructure lipid carriers and chemical enhancers, incorporated into hydrogel, has been found to increase the solubility of drug, protect it from degradation, effectively deliver the MTX transdermally in experimental animal model of RA, maintain the therapeutic concentration of drug at the affected site and reduce the unwanted off-target/side effects (Garg et al. 2016). Higher expression of folic acid receptor by activated macrophages in RA has been utilised for synthesis of MTX-folic acid-lipid nanoparticles for enhanced delivery of MTX to these macrophages (Cheng and Lee 2017). Studies suggest that development of TNFα-siRNA nanoparticle formulation displays promising efficacy for treatment of arthritis in mouse model of collagen-induced arthritis that does not respond to MTX (Aldayel et al. 2018).

A recent report demonstrated conjugation of 2-(3-mercaptopropylsulfanyl)-ethyl-1,1-bisphosphonic acid (thiolBP) with distearoyl phosphoethanolamine polyethylene glycol and its incorporation into micelles and liposomes to create mineral binding nanocarriers as therapeutic agents. Such mineral binding carriers are used for systemic administration of drugs targeted for bone deposition (Wang et al. 2012). It was emphasised that dioleoyl trimethylammonium propane (DOTAP)-based cationic liposomes, attached to six repetitive sequences of aspartate, serine and serine, had higher affinity for surfaces undergoing bone formation, as compared to bone-resorption surfaces. Additionally, the report also mentions that pleckstrin homology domain-containing family O member 1 (Plekho-1) is a casein kinase-2 interacting protein-1, which negatively regulates the cellular process of bone formation but does not affect bone resorption. Lipid nanoparticles encapsulating Plekho-1 siRNA, functionalised with DOTAP, have been found to specifically deliver the siRNA to the bone formation surface to enhance the process of osteogenesis (Liang et al. 2015).

Some of the recent reports have demonstrated the role of phytoestrogens such as quercetin in stimulation of oestrogen receptors (ERα and ERβ) and their use in reducing health risk in postmenopausal women. Quercetin-loaded phytosome nanoparticles have been found to enhance anti-oxidative, anti-inflammatory and hypolipidemic activity of quercetin, thus resulting in its enhanced therapeutic efficacy (Abd El-Fattah et al. 2017).

10.3.2 Silk Fibroin

Silk fibroin is a natural, biocompatible, biodegradable and low-cost polymer obtained from cocoons of *Bombyx mori*, *Philosamia ricini* (eri), *Antheraea mylitta* (tasar) and *Antheraea pernyi* (tussah) (Shao et al. 2016). It has 5263 amino acid residues including lysine, alanine, serine, tyrosine and valine, contributing to approximately 95%. The tyrosine residues in silk fibroin have strong electron-donating properties that make this polymer a good template for biosynthesis of silver and gold nanoparticles (Shao et al. 2016). Being a natural polymer, silk is biodegradable and offers good permeation for oxygen and water but has a low compressive strength. Reinforcement of silk scaffolds with various silk structures, such as silk particles, silk microfibre and degummed fibre, HA and other metallic nanoparticles improves its mechanical properties (Behera et al. 2017). Silk fibroin has been cross-linked using various approaches involving ionising radiation such as (1) gamma rays, electron beam and ion beam, (2) chemical cross-linkers such as genipin and glutaraldehyde and (3) nitrate salts and enzymatic cross-linkers such as tyrosinase (Kim et al. 2017). Various methods such as lipid template fabrication, spray drying, self-assembly and oil emulsion have been used to fabricate negatively charged, round and low toxic silk fibroin particles for delivery of bioactive molecules to the specific site (Hassani Besheli et al. 2017). BMP2-loaded silk nanoparticles are capable of inducing ectopic bone formation (Shi et al. 2013).

Osteomyelitis is a severe condition associated with bone and joint infections and tissue suppurations, ultimately leading to delayed union and amputation. When infection advances, bone necrosis restricts the intravenous delivery of antibiotic to the site of infection due to the shortage of blood supply. Administration of vancomycin-loaded silk fibroin nanoparticles has been found to reduce the bone infections at the defect site (Hassani Besheli et al. 2017). Fabrication of biocomposite using these silk nanoparticles along with PCL nanoparticles, ascorbic acid and dexamethasone has been found to provide suitable osteogenic environment for differentiation of adipose-derived stem cells (Gandhimathi 2015). Incorporation of silk fibroin nanoparticles into PLLA has been found to increase the surface roughness and hydrophilicity of the PLLA scaffolds, resulting in enhanced affinity for albumin attachment and bone tissue regeneration (Chen et al. 2017a).

10.3.3 Alendronate

Alendronate (ALN) is a derivative of bisphosphonate and can chelate strongly with the calcium ion of HA. This unique property endows it with strong affinity and rapid adsorption to HA/bone. In addition, this pyrophosphate derivative also inhibits bone resorption at the site with high osteoclastic activity and therefore has been extensively used in the treatment of osteoporosis and other skeletal diseases with increased bone resorption. Non-polymeric alendronate nanoparticles, stabilised by Poloxamer F68, when administered as dry powder inhalers, have been found to exhibit anti-osteoclastic activity and prevent vertebral bone loss (Sultana et al. 2012).

10.3.4 Proteins

Nanoparticles of bovine serum albumin (BSA) stabilised with PEG and modified with PEI coating were found to be more biocompatible under in vivo conditions. Encapsulation of BMP2 in these coated nanoparticles was reported to possess osteoinductive activity in ectopic animal model after subcutaneous implantation (Zhang et al. 2010). Similarly, elastin-like nanoparticles were also explored for delivery of BMP2 and BMP14 for bone tissue engineering (Bessa et al. 2010). Chemically synthesised pentamer of VPAVG (poly VPAVG, Val-Pro-Ala-Val-Gly) was shown to assemble into spherical nanoparticles. Encapsulation of BMP2 in these nanoparticles has been demonstrated to increase the in vitro bioactivity of BMP2 (Machado et al. 2012). Some of the additional protein-based injectable nanoparticle-based formulations have also been developed for localised and continuous delivery of molecules. In this reference, introduction of thermosensitive polyphosphazene with hydrophobic isoleucine ethyl ester and hydrophilic PEG has been found to enhance the amphiphilicity and hydrophobic interaction with BMP2.

Substitution of carboxylic acid moiety to this backbone supported ionic interaction with BMP2. An aqueous solution of BMP2 and these dual-interacting polymeric nanoparticles transformed into hydrogel at higher temperature and exhibited significant bone regeneration at the site of injection (Seo et al. 2015).

Gelatin is a protein obtained either by acid hydrolysis (type A gelatin) or by partial alkaline hydrolysis (type B gelatin) of animal collagen. It is relatively low antigenic, biocompatible and biodegradable and therefore is generally recognised safe for extravascular administration. Due to the presence of modifiable amino and carboxylic functional groups that allow for covalent attachment of targeted moieties, gelatin is a preferred macromolecule for fabrication of nanoparticle/scaffold (Mekhail et al. 2016; Forero et al. 2017). Gelatin can be modified to increase its drug loading efficiency, degradation rate and release kinetics (Farbod et al. 2016). Impregnation of PCL nanofibres with gelatin nanoparticles has been found to mimic natural extracellular matrix and has a degradability window within the bone regeneration time frame (Binulal et al. 2012). Conjugation of gelatin nanoparticles with bone-targeting alendronate has been found to enhance its affinity to the mineralised surface (Farbod et al. 2016). Moreover, a four-component system consisting of alendronate sodium trihydrate, gelatin, gemini surfactant and DNA has been demonstrated to be a promising vector for targeted gene delivery to bone tissues. The alendronate-functionalised gelatin supports targeting of bone cells and also forms the core of nanoparticles (Mekhail et al. 2016).

10.3.5 Polysaccharides

Macrophages have been identified both as local and systemic amplifiers of RA due to their high abundance in the inflamed synovial membrane. Secretion of inflammatory cytokine TNFα plays a major role in progression of synovial damage. siRNA-mediated silencing of TNFα expression is a promising therapeutic strategy for RA treatment. Local delivery of anti-TNFα siRNA represents a promising approach for reducing joint inflammation by directly targeting the macrophages (Te Boekhorst et al. 2012). It has been observed that these activated macrophages selectively take up MTX-loaded dextran sulphate nanoparticles upon systemic administration via scavenger receptor class A-mediated endocytosis. These nanoparticles were found to accumulate in the inflamed joints and improve the therapeutic efficacy (Heo et al. 2017b). Mineralised nanoparticles composed of PEGylated hyaluronic acid as the hydrophilic shell, 5β-cholanic acid as the hydrophobic core and CaP as the pH-responsive mineral were observed to release MTX due to pH-dependent demineralisation. Such nanoparticles were taken up by macrophages via receptor-mediated endocytosis, which involved molecular redundancy among different hyaladherins, including CD44, stabilin2 and receptor for hyaluronan-mediated motility (RHAMM) for effective drug delivery into arthritic tissues (Alam et al. 2017).

10.3.5.1 Chitosan

Chitosan is a copolymer of glucosamine and N-acetylglucosamine (Forero et al. 2017). It is a naturally occurring polysaccharide obtained by deacetylation of chitin. Since it is more soluble in aqueous and organic solvents than chitin, it potentially has collagen-replacing characteristics. At pH above 6.5, chitosan is poorly soluble and starts losing its cationic nature. Chitosan is a flexible polymer and lack the mechanical properties required for hard tissue engineering (Nazeer et al. 2017). *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) has been reported to possess excellent water solubility over a wide pH range. HTCC is prepared by replacing the original amino group by methyl group that prevents generation of hydrogen bonds by amino and hydroxyl groups, causing the solubilisation of chitosan in neutral and alkaline conditions. HTCC can be used as an absorption enhancer across intestinal epithelium due to its mucoadhesive and permeability-enhancing property (Zhao et al. 2010). Surface modification of chitosan with PEG has been reported to increase the stability of chitosan-parathyroid hormone (PTH) nanoparticles in gastric pH and prolong the circulation time in biological fluids. The exposed amine group of chitosan-PTH nanoparticles makes it prone to higher dissolution at gastric pH that eventually causes haemolysis due to increased concentration of chitosan (Narayanan et al. 2013).

Photopolymerisable hydrogel incorporating rhBMP2-loaded 2-N,6-O-sulphated chitosan nanoparticles is an electrostatic nanoparticle assembly that enhances the bioactivity of BMP2 for repair of bone defects (Cao et al. 2014). These sulphated chitosan nanoparticles loaded with BMP2 and incorporated into gelatin have been shown to promote angiogenesis and improve vascularisation during orthotropic bone formation (Nazemi et al. 2014). Minocycline-loaded chitosan nanoparticles used to prepare asymmetric collagen/chitosan-guided bone regenerative membrane have been found to promote adhesion of osteoblast and inhibit bacterial colonisation at the same time (Ma et al. 2016).

Some of the recent reports suggested that aspirin may also play an important role in modulating the balance between bone resorption and bone formation in osteoporotic animals. They showed that aspirin-loaded chitosan nanoparticles were capable of promoting bone regeneration without invasion of soft tissue. The release of aspirin was found to continue till 14 days due to diffusion and polymer degradation (Zhang et al. 2017a). Raloxifene is a second-generation non-steroidal benzothiophene that acts as a selective oestrogen receptor modulator for prevention and treatment of postmenopausal osteoporosis. Raloxifene gets rapidly absorbed in the gastrointestinal tract and undergoes extensive glucuronidation, which reduces its bioavailability. Few of the reports demonstrated that encapsulation of raloxifene in mucoadhesive chitosan nanoparticles and its administration through nasal route increased the retention time and enhanced its absorption at the site of administration (Zhang et al. 2017a). Incorporation of rosuvastatin-loaded chitosan/chondroitin sulphate nanoparticles into thermosensitive Pluronic F127/hyaluronic acid hydrogel

has been shown to achieve localised (injectable) and controlled delivery of rosuvastatin to bone tissues (Rezazadeh et al. 2018).

Fabrication of positively charged chitosan, negatively charged oxidised sodium alginate and positively charged BSA-based biomimetic extracellular matrix nanostructures, through layer-by-layer self-assembly, has been demonstrated to preserve the activity and sustained release of BMP2. It was suggested that covalent bonding between aldehyde group of oxidised sodium alginate and amino group of chitosan improves the stability and therefore promotes cellular attachment, proliferation and differentiation of MSCs (Wang et al. 2016b). BSA microparticles, encapsulating chitosan-dextran sulphate nanoparticles and MSCs, were used as dual-delivery system for bone regeneration (Valente et al. 2013).

The applications of microRNAs in bone regeneration have been limited due to their poor stability, low cellular uptake and undesired immune response. To address the shortcoming, some of the research groups synthesised chitosan/tripolyphosphate/hyaluronic acid nanoparticles for successful delivery of non-viral carrier of antimiR138 to bone marrow MSCs and showed significant enhancement in osteogenesis (Wu et al. 2016). Additionally, a dual-release system comprising of stromal cell-derived factor1α (SDF1α) and chitosan/tripolyphosphate/hyaluronic acid/antimiRNA-138 nanoparticles, in chitosan/β-sodium glycerol phosphate hydrogel, has been suggested as a potential therapeutic option for in situ hard tissue regeneration (Wu et al. 2018).

Lee et al. (2014) emphasised on the development of gene silencing approaches for treatment of RA. The study involved design of poly-siRNA/thiolated glycol chitosan nanoparticle system to target TNFα in arthritic joints. It was observed that this nanoparticle got internalised in activated macrophages and blocked the expression of TNFα. Since polymerised siRNA cannot freely cross the cell membrane, such a delivery system acted as a method of choice for nanoparticle-based delivery of gene silencing material in RA patients (Lee et al. 2014).

10.3.6 Poly(lactic-co-glycolic Acid) (PLGA)

PLGA is an FDA-approved polymer that has been used for a variety of biomedical applications for designing of drug delivery devices and surgical sutures. It exhibits excellent host biocompatibility, variable physico-chemical properties and predictable degradation rates. PLGA can be easily modified and functionalised by surface hydrolysis, aminolysis and O_2 plasma treatment to facilitate attachment of reactive groups such as carboxyl, amine, hydroxyl or peroxyl groups. This functionalisation supports covalent attachment of biological molecules to PLGA (Jiang et al. 2014).

Many of the previous reports have shown that the reaction between tetracycline and HA supports uptake of tetracycline by bone, and therefore tetracycline-based bone-targeting moieties have been designed. Esterification reaction between

hydroxyl group of tetracycline and carboxyl group of PLGA was utilised for synthesis of nanoparticles (Wang et al. 2015). Few of the reports have studied synthesis of bone-targeting nanoparticles using dendritic trimer in the aspartic acid oligopeptide (Asp3) conjugated to PLGA-PEG copolymers (Fu et al. 2014). Similarly, polyaspartic acid peptide sequence has been demonstrated to preferably recognise resorption sites in skeletal tissues due to ionic interactions between negatively charged peptide sequence and positively charged calcium ions within the bone at physiological pH. Therefore, aspartic acid peptide-conjugated PLGA nanoparticles are favoured for targeted delivery to osteoporotic sites (Jiang et al. 2014).

PLGA nanoparticles have been used for fabrication of cohesive colloidal gel that is used as an injectable filler to promote healing of bone defects (Wang et al. 2010). PLGA nanoparticles have also been incorporated into porous scaffolds composed of chitosan and gelatin blend cross-linked with genipin. Such a scaffold has been found to act as local regulator to control doses and kinetics of released growth factors, thus increasing their potential retention time at therapeutic concentration levels (Gentile et al. 2015). A novel calcification-targeting nanoparticle has been synthesised through dopamine self-polymerisation on the PLGA particle surface and subsequent alendronate conjugation (Li et al. 2016b).

Fabrication of N-hydroxysuccinimide (NHS)-modified PLGA nanoparticles with mussel-inspired alginate-dopamine polymer has been found to increase the tissue adhesive properties (Pandey et al. 2018). PLGA nanoparticles loaded with anti-TNFα-siRNA was shown to significantly decrease the paw scores and joint effusions in collagen-induced arthritic mice (Te Boekhorst et al. 2012). Dexamethasone-loaded biphasic CaP granules incorporated into PLGA nanoparticles have also been suggested to serve as efficient drug-releasing nanocarriers (Son et al. 2015).

10.4 Conclusion

Nanoparticle-based approaches for targeted drug delivery, fabrication of scaffolds and other therapeutic applications have gained tremendous value during the last decade, due to its efficacy in the biomedical field. But there might be challenges associated with the nano-safety issues. These particles may initially seem to be safe, but their accumulation either in biological systems or in the environment over a period of time might have grave consequences. Therefore, it becomes essential to understand the molecular mechanisms associated with the toxicity of these nanoparticles. The in vitro and in vivo screening methods have to be elaborate so as to interpret any long-term damage that could be associated with these particles, with specific emphasis on the determination of their in vivo dosages. Thus, the major challenge for nanomaterial safety assessment is to develop extensive methodologies for handling and screening of large number of newly engineered nanomaterials (Gross 2015).

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Chapter 11
Polyphenol-Based Nanoparticles as Multifaceted Diabetes Modulators

Raquel Costa-Almeida, Raquel Soares, and Raquel Costa

Abstract Type 2 diabetes is am chronic disease characterized by insulin resistance, which results in extensive metabolic dysfunction and in a large number of chronic complications. Microangiopathy, macrovascular disease, immune dysfunction, and endothelial dysfunction, among many other conditions, are responsible for high morbidity and mortality rates. The wide range of biological properties of natural polyphenols renders these compounds excellent agents for diabetic complications treatment as revealed in the literature. Nevertheless, these beneficial effects are often mitigated by the chemical properties of these molecules, which result in reduced stability, bioavailability, digestion, intestinal absorption, cell uptake, and pH sensitivity, among other features. Herein, an overview of the nanotechnology and delivery systems currently developed to overcome these drawbacks will be presented. In particular, key criteria to be fulfilled by nanosystems for encapsulating polyphenols are highlighted. Strategies to improve polyphenol bioavailability are discussed, and special attention is given to nanoparticle properties influencing cellular uptake, including size, surface charge, and shape, as well as to polymers from both natural and synthetic origin that can enhance the bioactivity and efficacy of delivered polyphenols. The application of smart engineering of polyphenol-loaded nanosystems is a promising tool for the treatment of the wide range of complications observed in diabetes.

Keywords Bioavailability · Diabetes · Drug delivery · Metabolic dysfunction · Metabolism · Nanoencapsulation · Polyphenols · Diabetic vascular complications

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_11

11.1 Background

Type 2 diabetes (T2D) is a chronic metabolic disease rapidly rising and a major health concern worldwide. Although the underlying causes of metabolic dysfunctions and vascular complications are multifactorial, insulin resistance and hyperglycemia are the main driving forces. Additionally, oxidative stress, chronic low-grade inflammation, and endothelial dysfunction also play a central role in the progression of T2D.

In the last decades, there has been increasing evidence that dietary polyphenols, among the well-known antioxidant behavior, are able to modulate vascularization and inflammation and regulate metabolic pathways, preventing T2D or mitigating its symptoms. As a result of its multifunctional properties, polyphenols have emerged as promising candidates for hampering chronic diseases, namely, obesity and T2D (Dominguez Avila et al. 2017; Tresserra-Rimbau et al. 2017; Fraga et al. 2019). Literature based on in vitro and in vivo studies points out that the most challenging issue for developing polyphenols as antidiabetic agents is related with low oral bioavailability (Dominguez Avila et al. 2017). This property may be the major reason relating to its ambiguous therapeutic effects and large interindividual variations in clinical trials.

Thus, an urgent need exists to develop novel strategies toward improving polyphenol absorption and, consequently, their bioavailability. Nanotechnological advances have been driving the refinement of drug delivery systems with promising outcomes regarding the encapsulation of polyphenols. Furthermore, through adequate modifications, nanosystems can guide polyphenols to target sites, leading to locally increased concentrations of the bioactive compound. In this chapter, we discuss some innovative nanotechnological applications to improve polyphenol function and their mechanism of action in preventing T2D.

11.2 Polyphenols

11.2.1 Bioavailability and Health Benefits of Polyphenols

Polyphenols are naturally occurring compounds largely found in a wide range of foods consumed in Western diets. They are present in fruits, vegetables, cacao, and beverages, such as wine, tea, and beer. Until now, more than 8000 phenolic compounds have been identified (Manach et al. 2005). Phenolic compounds are characterized by the presence of hydroxyl groups covalently linked to an aromatic ring (Manach et al. 2004). They are classified according to the number of phenol units they contain and its properties and distributed in subclasses such as flavonoids, stilbenes, phenolic acids, and lignans (Manach et al. 2005). Thus, polyphenols have

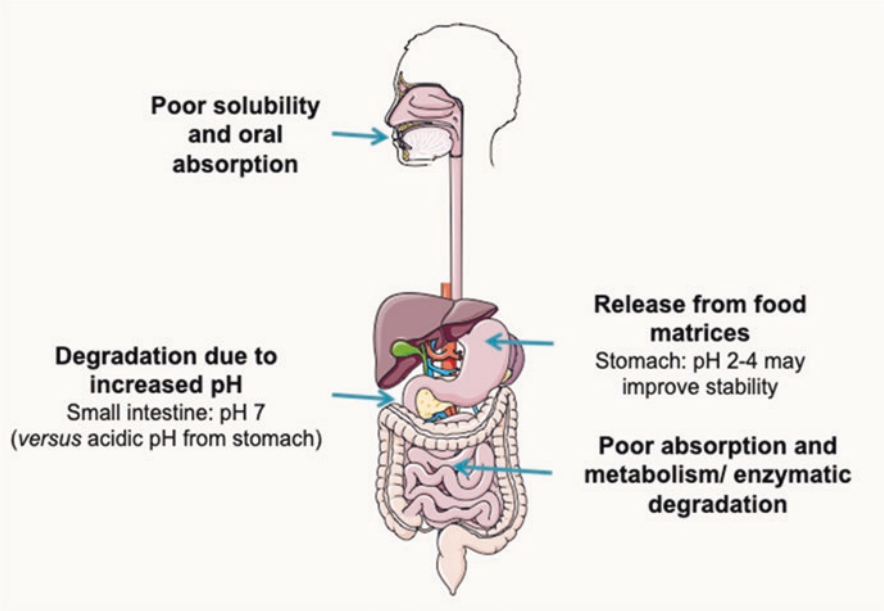

Fig. 11.1 Schematic representation of multifactorial parameters contributing for the limited bioavailability of polyphenols

different chemical structures and solubilities resulting in various biological properties (Manach et al. 2004; Gothai et al. 2016). This difference in their chemical constitution, solubility, the amount of polyphenol consumed, and the interaction with food matrices influences their bioavailability (Fig. 11.1), which is the ratio of the absorbed polyphenol detected in the target site compared to the total orally ingested amount (Mena and Llorach 2017). It is important to note that the bioavailability of polyphenols differs greatly from the bioactivity tested in vitro; the most abundant diet-derived polyphenols or the highest bioactive compound tested in human cell culture does not necessarily correlate with the most absorbed and metabolized by the human body (Mena and Llorach 2017). Their bioavailability exhibits inter-individual variations, due to alterations in digestion, intestinal absorption, metabolic rate, and biological action in target tissues, exerting a wide range of biological activities (Manach et al. 2005; Mena and Llorach 2017). It is difficult to recommend dietary reference intake levels for polyphenols as they are considered non-nutrient compounds and due to personal variations after intake, but it is known that the average daily polyphenol consumption is approximately 1 g per person (Manach et al. 2005).

In nature, polyphenols could be stored in plants as glycoside or non-glycoside conjugates, as secondary metabolites, which influence their absorption and thus their bioavailability (Gothai et al. 2016). The bioavailability of polyphenols is influenced by various external factors, including food processing and storage conditions (temperature, oxygen, light) and interactions with food matrices, as well as by host conditions, involving intestinal and systemic factors (D'Archivio et al. 2010; Gothai et al. 2016).

Polyphenols are mainly absorbed in the small intestine being metabolized before entering the bloodstream to be delivered to different tissues and organs to exert their biological activities. However, a significant amount of the unabsorbed phenols and those bound to cell wall materials enter the colon and are transformed by the intestinal bacteria (Selma et al. 2009; Etxeberria et al. 2013). For this reason, the colon is considered an active site for metabolism of polyphenols since gut microbiota is able to transform the ingested compounds into their metabolites by hydrolyzing glycosides, glucuronides, sulfates, amides, esters, and lactones, being also capable of carrying out ring cleavage, reduction, decarboxylation, demethylation, and dehydroxylation reactions (Etxeberria et al. 2013; Anhe et al. 2015). However, there is a two-way interaction since polyphenols have been also demonstrated to modulate gut microbiota composition owing to their antimicrobial properties (Selma et al. 2009; Anhe et al. 2015; Murota et al. 2018). Since gut microbiota composition is distinct from one person to another, the polyphenol metabolites present in plasma, feces, and urine exhibit interindividual behavior, and it may be linked to distinct health outcomes (Crozier et al. 2010).

The current knowledge advocates that polyphenols have pleiotropic effects on biological systems, being able to mitigate chronic diseases as obesity and T2D. The main mechanism of action of these compounds was primarily related to their direct antioxidant capacity. But they induce, simultaneously, other beneficially health effects, namely, anti-inflammatory, anticancer, neuroprotective, as well as regulatory effects of multiple biochemical, metabolic, and endocrine pathways and improvement of gut health (Stevenson and Hurst 2007; Selma et al. 2009; Mena and Llorach 2017; Fraga et al. 2019). In recent years, the antidiabetic potential of polyphenols has represented a major focus of basic and clinic research (Bahadoran et al. 2013).

Based on the observed health-promoting benefits and the advantages of its novel formulations based on micro- and nanomaterials, polyphenols have emerged as a potential alternative and additive therapy for the wide variety of diabetic complications. Efforts made in innovative encapsulation strategies to improve bioavailability and efficiency of polyphenols will be a promising approach in preventing noncommunicable diseases overall.

11.3 Nanotechnology and Polyphenols

The contribution of polyphenols in regulating several biological processes renders these biomolecules of interest for applications in human health, ranging from anti-aging and skin photoprotection (Nichols and Katiyar 2010; Afaq and Katiyar 2011),

cancer chemoprevention (Tabrez et al. 2013), and cardio- and neuroprotection (Pandey and Rizvi 2009) to the modulation of diabetes and associated complications. The limited stability, poor oral absorption and bioavailability, and rapid metabolic transformation have been driving the development of nanodelivery systems toward overcoming these drawbacks and enhancing the therapeutic potential/efficacy of polyphenols (Santos et al. 2013; Puligundla et al. 2017; Hu et al. 2017).

11.3.1 Nanoencapsulation Methods

Several chemical, physical/mechanical, and physicochemical methods exist for the nanoencapsulation of polyphenols. Different polyphenols and target applications may require different processing and fabrication technologies, and detailed reviews can be found in the literature (Mukhopadhyay and Prajapati 2015; Rakotoarisoa and Angelova 2018; Pannu and Bhatnagar 2019; Santos et al. 2019). Herein, a brief overview on the classification of different processes is provided, since nanoencapsulation techniques have been reviewed in detail elsewhere (Reis et al. 2006; Wais et al. 2016).

Chemical methods for encapsulating bioactive molecules are bottom-up techniques that require the addition of a cross-linking agent to promote the polymerization of monomers at the interface of two immiscible substances—the active compound and the matrix in which biomolecules of interest, namely, polyphenols, will be entrapped (Reis et al. 2006; Conte et al. 2016). These methods include polyelectrolyte complexation and interfacial and in situ polymerization. Chemical nanoencapsulation yields nanoparticles with high purity and uniformity, small particle size with a narrow size distribution, dispersibility, and increased possibilities for the introduction of functionalities through the existence of defined reactive groups.

Nanoencapsulation by physical or mechanical means relies on the interactions between the vehicle material and the biomolecule to be encapsulated when both are atomized or aerosolized. These methods include spray drying, electrospraying, solvent evaporation, and pan coating, among others (Wais et al. 2016). Nonetheless, top-down physical/mechanical methods as wet-milling and high pressure homogenization, which are mostly used at the industrial level owing to their scale-up potential, present limitations regarding the production of small, uniform, and non-aggregated nanoparticles (Wais et al. 2016).

Finally, combining both physical and chemical techniques, physicochemical processes, like phase inversion nanoencapsulation (PIN), coacervation, and phase separation, resulted in the formation of stable nanometer size drug nanosuspensions or nanoparticles. These processes have been widely explored given their ability to increase loading capacities and persistence at the target sites.

Overall, nanoencapsulation of polyphenols for mediated/triggered delivery has been investigated using different nanostructures, including cyclodextrins, nanospheres, nanocapsules, solid lipid nanoparticles (SLN), liposomes, and micelles (Conte et al. 2016).

11.3.2 Nanotechnology for Improved Bioavailability and Efficacy of Polyphenols

The low bioavailability of polyphenols is mainly associated to pre- and postabsorptive metabolism in the gastrointestinal (GI) tract, as discussed above. Hence, delivery systems are widely investigated toward overcoming these drawbacks and enhancing the therapeutic potential/efficacy of polyphenols (Santos et al. 2013; Hu et al. 2017; Puligundla et al. 2017). In particular, delivery systems should satisfy a number of requirements, including the ability (1) to maintain the bioactive form of the polyphenol, (2) to protect the encapsulated compound(s) from the upper GI tract and release in the colon (pH sensitive behavior), and (3) to release the compound in a site-specific and controlled/sustained manner (Mukhopadhyay and Prajapati 2015). Nanoencapsulation may increase the levels of polyphenols through different mechanisms, namely, through improving oral absorption, enhancing gastric residence time through mucosal adhesion, avoiding the first-pass effect of pre-systemic hepatic metabolism, or increasing cellular uptake at the target tissue (Cai et al. 2010; Punfa et al. 2012; Lesniak et al. 2013; Ndong Ntoutoume et al. 2016; Phan et al. 2016; Siu et al. 2018; Chimento et al. 2019). Chemical interactions between polyphenols and nanoparticles (e.g., hydrogen bonds, hydrophobic interactions) enable the entrapment of these bioactive compounds, minimizing enzymatic and nonenzymatic degradation, consequently resulting in prolonged circulation half-life and accumulation (Cai et al. 2010; Li et al. 2015; Puligundla et al. 2017).

Nanoparticle sizes range between 1 and 100 nm, but evidences support that nano-to-microparticles with 100–1000 nm can also result in enhanced bioavailability of nutrients and nutraceuticals (Acosta 2009). Most frequently, this effect occurs as a result of direct uptake of the nanoparticle. Therefore, issues related to size, shape, surface charge, and surface chemistry of the nanodelivery vehicle must be considered (Murugan et al. 2015). Fine-tuning these physicochemical characteristics will enable an active targeted intracellular delivery of the biomolecule-loaded nanosystem, as well as a control over cellular uptake. Remarkably, a concentration-dependent effect on epithelial cell viability has been demonstrated for positively charged nanoparticles with sizes of 50 and 100 nm, but not for negatively charged ones (Bannunah et al. 2014). Differences in electrostatic interactions between particles and cell membrane may be the basis for this disparity in terms of toxicity, given that negatively charged nanoparticles are electrostatically repelled from the cell membrane (Nangia and Sureshkumar 2012). Additionally, shape greatly

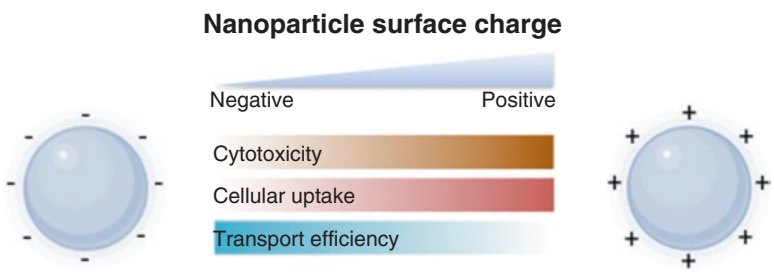

Fig. 11.2 Surface charge-dependent effects on intestinal epithelial cells

influences cellular uptake and translocation of nanoparticles. Indeed, positively charged nanoparticles with anisotropic morphologies can reorient in the extracellular environment, thereby enhancing the contact area with cell membrane and disrupting the lipid bilayer, being easily internalized (Nangia and Sureshkumar 2012).

Furthermore, it is worth to note that strategies to improve the intestinal absorption of polyphenols (and of other molecules/drugs) should take into consideration the particular characteristics of intestinal epithelium, which exhibits different internalization and transport capacities. Polarized epithelial cells have been demonstrated to interact differently with nanoparticles and to use different endocytic mechanisms according to the apical or basolateral side of the cell (Apodaca 2001; Xu et al. 2012). Surface charge, rather than particle size, has been described as a critical parameter mediating the interactions between intestinal epithelial cells and nanoparticles (Fig. 11.2), including cellular uptake and translocation as different internalization pathways can be involved (Bannunah et al. 2014). After being internalized by enterocytes, cationic nanoparticles also provide protection against endolysosomal degradation.

11.3.2.1 Polymers for Nanoencapsulation of Polyphenols

The selection of an adequate material can contribute substantially toward enhancing the bioavailability of polyphenols and possibly leading to potentiating/synergistic biological effects. Successful delivery of polyphenols requires nanocarriers to fulfill the traditional requirements for applications in human health: biodegradability, biocompatibility, as well as lack of cytotoxic and immunogenic effects. Polymers from both natural (e.g., chitosan and its derivatives, alginate, pectin, carrageenan, xanthan gum, cellulose, gelatin, albumin, and casein, among others) and synthetic origins (e.g., polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone (PCL), among others), as well as natural/synthetic polymer combinations, can be explored for encapsulating polyphenols (Paini et al. 2015; Hu and Luo 2016; Sanna et al. 2016; Sarika et al. 2016; Hu et al. 2017; Prasad et al. 2017).

Owing to its unique properties as a natural origin polymer, chitosan is commonly used for nanoencapsulation of polyphenols (Hu and Luo 2016). The cationic nature renders this biopolymer of potential to strongly bind negatively charged molecules and, thus, to be widely explored in polyelectrolyte complexation processes (Hu and Luo 2016; Costa-Almeida et al. 2017). Chitosan is a linear polysaccharide obtained from chitin and consisting of repeating units of β-(1 → 4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. It has mucoadhesive features, which result in prolonged residence time in the mucosa and consequently increased bioavailability of bioactive compounds or drugs (Ways et al. 2018). This effect is generally explained by the interactions between chitosan positive charges and negatively charged components of the gastric mucus, as *N*-acetylneuraminic acid, which enable a prolonged contact between delivered compound and epithelia, thus improving drug diffusion (Lin et al. 2015). Furthermore, different functionalization strategies can be pursued owing to the existence of hydroxyl and amine groups in chitosan backbone, enabling the development of nanocarriers with specific targeting. For instance, the encapsulation of epigallocatechin-3-gallate (EGCG) in nanoparticles composed of fucose-conjugated chitosan and polyethylene glycol (PEG)-conjugated chitosan/gelatin appeared to efficiently target gastric cancer cells, owing to increased fucosylation inside cancer cells, resulting in a site-specific release of the entrapped polyphenol (Lin et al. 2015). Additionally, the entrapment of EGCG within nanoparticles prepared through ionic gelation of a chitosan derivative (chitosan-tripolyphosphate) improved oral absorption, as well as intestinal stability, leading to an overall increase in EGCG plasma levels by a factor of 1.5 relative to free EGCG solution (Dube et al. 2011).

Furthermore, alginate, a natural origin polysaccharide derived from brown seaweed, is a well-known pH-sensitive polymer (Jain and Bar-Shalom 2014). Indeed, by shrinking under low pH values, alginate allows for retaining the encapsulated polyphenol rather than releasing it in the stomach, protecting the compound against enzymatic degradation and enabling a targeted release according to changes in pH conditions (Lin et al. 2015).

Another natural polymer with interesting features is guar gum, a nonionic hydrophilic polysaccharide with a swelling behavior in cold water, forming viscous colloidal dispersions, and that has been described as a colon-specific drug delivery vehicle (Hongbo et al. 2012). Its gelling capacity enables a control over drug release in the upper GI tract, favoring degradation in the colon (Mukhopadhyay and Prajapati 2015), avoiding first-pass metabolism, and consequently enhancing polyphenol bioavailability.

Similarly, synthetic polymers are being explored as nanoencapsulation systems given their promising features for industrial scale-up production, including the reduced batch-to-batch variations, easier synthesis in large quantities, and control over the polymeric structure (Englert et al. 2018). For instance, nanoparticles produced from galactosylated PLGA, a synthetic origin polymer with slow degradation rate, could promote intestinal stability of resveratrol and favor its transport through intestinal epithelia, simultaneously boosting the anti-inflammatory activity of resveratrol (Siu et al. 2018). More detailed examples on antidiabetic applications are discussed in the following section.

11.4 Applications of Polyphenol Nanosystems in Diabetes

11.4.1 Metabolic Modulation

Sustained insulin resistance, hyperglycemia, and hyperlipidemia observed in T2D lead to impairment of several metabolic pathways and contribute to the pathogenesis of the disease. The available pharmaceutical treatments for T2D management focus on glucose-lowering agents that maintain blood glucose concentration within the physiological range (DeFronzo et al. 2015). Despite intense research, these approaches are not completely efficient as they have limited efficacy, safety, and cost-effectiveness or present undesirable side effects (Tahrani et al. 2016).

Recent studies have reported the antidiabetic potential of polyphenols as they exert a glucose-lowering effect through mechanisms similar to those exerted by currently used pharmaceutical drugs (Bahadoran et al. 2013; Costa et al. 2017; Dominguez Avila et al. 2017). It is urgent to unravel the mechanism by which polyphenols regulate metabolic pathways and how could it be ameliorated by polyphenol encapsulation, to provide guidance for T2D prevention and treatment.

Polyphenols are able to prevent or mitigate T2D-related metabolic complications by inhibiting glucose intestinal absorption, controlling carbohydrate metabolism, protecting pancreatic β-cell damage, and improving pancreatic insulin secretion and insulin sensitivity in peripheral tissues (Bahadoran et al. 2013; Zhao et al. 2013).

Dietary polyphenols are able to inhibit the activity of key metabolic enzymes of carbohydrate digestion, such as α-amylase and α-glucosidase. However, there are some undesirable effects due to oral administration of polyphenols given the nonspecific and noncompetitive interactions with other proteins, which lead to diminished enzymatic activity in the gastrointestinal tract. Many efforts have been made in order to counteract these drawbacks with successful results. A pH-sensitive delivery system based on micron-sized calcium-alginate beads was developed in order to protect polyphenols (e.g., tannic acid) from proteins in the stomach, allowing a targeted release in the small intestine inhibiting α-amylase activity, as thus, carbohydrate digestion (Zhao et al. 2013). They are also able to stimulate the secretion of glucagon-like peptide 1 (GLP-1), an insulinotropic hormone poorly secreted by diabetic individuals, from intestinal L-cells (Dominguez Avila et al. 2017). The antihyperglycemic effect of GLP-1 occurs through direct stimulation of synthesis and secretion of insulin by β-cells. Additionally, in these cells, it improves glucose sensitivity, has antiapoptotic effect, stimulates proliferation and differentiation, and inhibits glucagon secretion by pancreatic α-cells (Moonschi et al. 2018). However, native GLP-1 therapy is inappropriate due to the rapid post-secretory inactivation by DPP-4. Recently, it has been reported that the oral delivery of GLP-1 was facilitated through loading in nanoparticles coated with chitosan for mucoadhesiveness and further entrapped within a pH-responsive polymeric matrix. The nanosystem with a final size of approximately 600 nm was able to cross the intestinal epithelium, improving the GLP-1 permeability (Martins et al. 2018). Another interesting application of chitosan, in the form of chitosan oligosaccharide (COS) with higher water

solubility and lower viscosity, more easily absorbed through the intestine, and safety profile holds therapeutic promise in T2D. COS has favorable pharmacological properties and displays important biological activities, namely, the activation of AMP-activated protein kinase (AMPK), a master metabolic regulator and sensor of cellular energy status (Liu et al. 2010; Chiu et al. 2015; Mattaveewong et al. 2016). The development of COS loaded with polyphenols may be of useful interest for potentiating their effect in target tissues through synergistic effects mediated by chitosan and polyphenols. The activation of AMPK is the main target of some anti-diabetic drugs, and it has been reported that polyphenols activate AMPK to an extent 50–200 times higher than metformin (Zang et al. 2006). By activating AMPK, polyphenols affect several metabolic pathways, switching on catabolic pathways, namely, fatty acid oxidation and glycolysis, and switching off ATP-consuming pathways, namely, lipogenesis (Dermaku-Sopjani and Sopjani 2019).

In peripheral tissues, such as muscle and adipocytes, polyphenols such as quercetin, resveratrol, epicatechin, EGCG, grape seed-derived procyanidins, xanthohumol, and 8-prenylnaringenine from beer, blueberries, and black soybean could improve insulin-dependent glucose uptake by translocation of GLUT-4 to the plasma membrane through the induction AMPK (Park et al. 2007; Vuong et al. 2007; Montagut et al. 2010; Bahadoran et al. 2013; Kurimoto et al. 2013; Ueda-Wakagi et al. 2015; Costa et al. 2017; Kim et al. 2018). In coordination with peripheral tissues, liver plays an important role in the regulation of blood glucose levels. Regarding hepatic metabolic pathways, there are novel discoveries demonstrating the use of nanoparticles composed of modified chitosan/polyethylene glycol (CT/PEG) conjugated with glycyrrhetinic acid (GA) with spherical morphology and ca. 189 nm in size to improve their liver uptake due to the abundance of GA receptors at the cell membrane of hepatocytes. With this CT/PEG-GA, Rastegari et al. (2019) demonstrated a promising approach to control glucose levels by diminishing the expression of gluconeogenic genes using siRNA technology for silencing hepatic cAMP response element binding protein (CREB)-regulated transcriptional coactivator 2 (*CRTC2*), which is overexpressed in T2D patients.

11.4.2 Anti-inflammatory and Antioxidant Potential of Encapsulated Polyphenols

A state of chronic inflammation and oxidative stress is implicated in the pathogenesis and progression of T2D and its complications. Patients with diabetes display typical features of an inflammatory process characterized by the increased expression of C-reactive protein and cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), together with the activation of several immune reactions (Gothai et al. 2016; Fraga et al. 2019). This state of chronic inflammation is also attributable to oxidative stress, with an imbalance between oxidants and antioxidants potentiating insulin resistance and β-cell

dysfunction through the activation of stress-sensitive signaling pathways (Arulselvan et al. 2016), being both processes particularly involved in the pathogenesis of obesity-linked insulin resistance and T2D. A huge list of anti-inflammatory drugs is prescribed to diabetic patients. Yet, these comprise several secondary complications, namely, dizziness, metabolic disorders, cardiovascular, and liver and kidney failure disease. Therefore, identifying bioactive molecules derived from natural products that exert anti-inflammatory effects is fundamental. Numerous researches have proven that polyphenols exert their biological properties by blocking signaling pathways such as NF-κB and mitogen-activated protein kinases (MAPKs) which have the main role in the production of various pro-inflammatory mediators (Santangelo et al. 2007). They are able to modulate the expression of several pro-inflammatory molecules such as cytokines, lipoxygenase, nitric oxide synthase, and cyclooxygenase. In addition, polyphenols neutralize undesired reactive oxygen species (e.g., hydroxyl radicals, superoxide anion radicals, and hydrogen peroxide) and nitrogen species produced as byproduct during metabolic processes and increase endogenous enzymatic and nonenzymatic defenses. Besides having antioxidant activities and restoring the redox balance to reduce oxidative stress, polyphenols may prevent chronic inflammation through mitigation of cytokine pathways and regulation of inflammatory signaling (Santangelo et al. 2007; Arulselvan et al. 2016).

Through the decades, several in vitro and in vivo studies provide strong evidence that polyphenols, namely, xanthohumol, catechins, EGCG, curcumin, and resveratrol, exert antioxidant and anti-inflammatory effects in diabetic-related metabolic alterations (Stevenson and Hurst 2007; Costa et al. 2013; Siu et al. 2018). Despite these health benefits, pharmacokinetic studies demonstrate a low bioavailability, solubility, absorption, and metabolism after oral administration of polyphenols along with short life raising some drawbacks in its general use. Lu et al. (2013) developed a resveratrol-loaded nanoparticle based on polyvinylpyrrolidone polymer and reported its biocompatibility and uptake in neuron cultures. This and other in vitro studies revealed that nanoparticles with antioxidant properties may improve vascular dysfunction associated with hypertension, diabetes mellitus, or atherosclerosis (Mauricio et al. 2018).

Moreover, a recent work conducted by Siu et al. (2018) demonstrates the benefits of alginate nanoparticles, combined with galactose, as an oral delivery vehicle for resveratrol. The galactose moiety acts as an anchor to promote intestinal absorption of polyphenols through sodium glucose transporter-1 (SGLT1) at the apical cell membrane, by increasing the affinity and specificity of NP for enterocytes, when compared to conventional NP. This study was performed in animals and LPS-induced macrophages, and they showed that this nanocarrier promotes intestinal absorption of resveratrol and its physiological stability and thus enhances its bioactivity and anti-inflammatory response, by suppressing TNF-α, IL-6, and nitric oxide (NO) release (Siu et al. 2018).

Other interesting conjugation relies on NPs based on metal oxide, such as cerium oxide (CeO_2) or yttrium oxide (Y_2O_3), and those that are carbon-based, such as fullerenes, that have been developed with radical-quenching and catalytic properties. Ciofani et al. (2014) demonstrate that cerium nanoparticles were able to

scavenge ROS in neuron-like PC12 cells, diminishing 25–50% of intracellular ROS when cells are exposed to H_2O_2. These NPs can be used to encapsulate polyphenols and further enhance the ROS scavenger activity, providing a novel strategy to counteract oxidative stress in T2D. Nevertheless, a long way is needed until these agents can be used in clinical studies addressing diabetes.

11.4.3 Vascular-Related Diabetic Complications

Vascular disease in diabetes affects both macro- and microvessels, being responsible for the huge morbidity and mortality rates of diabetic patients, as well as extensive healthcare management and costs. Diabetes-associated microvascular disease is mainly attributed to uncontrolled glycemia, although many other factors also contribute to these complications, namely, insulin resistance, hypertension, dyslipidemia, and smoking (Beckman and Creager 2016). Among these microvascular complications, retinopathy is the most common condition observed in diabetic patients. Recently, intravitreal administration of anti-vascular endothelial growth factor (VEGF) agents has been shown to improve outcomes in proliferative retinopathy. Nevertheless, patient adherence to the intravitreal or topical treatment is a leading cause of poor outcomes. To overcome this, many nanosystems, targeting the retina and other internal eye tissues, have already been designed, offering effective solutions.

Preclinical studies have addressed the use of siRNA against proteins that are overexpressed in retinopathy complexed with lipid-based nanocarriers and injected in the eye of streptozotocin (STZ)-induced diabetic rats (Amadio et al. 2016). The siRNA was efficiently delivered within internal eye tissues, ultimately downregulating VEGF and exerting, hence, benefic effects.

The anti-inflammatory, antioxidant, anticancer, and cardiovascular protective actions of polyphenols, described above, render these compounds promising agents to target diabetic microvascular disease. Our group has previously studied the effect of isoxanthohumol, a metabolite obtained from beer-derived xanthohumol, in the retina vascularization using pup retina assays (Negrao et al. 2013). We were able to show that i.p. administration of isoxanthohumol resulted in a 20% inhibition of sprouting angiogenesis, also affecting leukostasis and inflammation in the retina. These findings highlight the putative role of these natural compounds in controlling diabetic retinopathy.

Polyphenols have recently been applied to these nanotechnology approaches. One example is resveratrol, a plant-derived polyphenol with well-established antioxidant agents, also playing a role in treatment and prevention of diabetic complications (Popescu et al. 2018). Using the STZ-induced diabetic rat model previously described, Dong et al. (2019) synthesized gold nanoparticles coated with resveratrol, which acted as a stabilizing and reducing agent. Oral administration of the gold nanoparticles to these animals improved diabetic retinopathy features, namely, decreasing retinal vascular permeability, angiogenesis, and inflammation. According

to these authors, gold nanoparticles are able to carry higher amounts of resveratrol and efficiently deliver it inside the cells.

Kidney disease is another drastic complication of diabetes, often associated with worse prognosis in diabetic patients and ultimately resulting in end-stage renal failure. Here too, natural compounds, such as EGCG, curcumin, oligonol, or resveratrol, have been reported to play a role as protective agents (Liu et al. 2016; Phyu et al. 2016; Sun et al. 2017; Kanlaya and Thongboonkerd 2019). These effects have been described in preclinical assays only. Although polyphenols have already been used in clinical trials, most of these address cancer or neurodegenerative disease. Moreover, alternative drug delivery systems, such as lipid nanocapsules, exosomes, nanocomposites, and emulsified formulations or in gel form, are underway in rodent models (Renaud and Martinoli 2019).

Nanotechnology-based devices have been already described for diagnostic purposes targeting diabetic kidney disease (Kumar et al. 2018). Nonetheless, these strategies have not yet been used for therapeutic or preventive purposes.

Diabetic foot ulcers (DFU) are a late-stage complication of diabetes mellitus, associated with huge morbidity and mortality rates (Loots et al. 1998). Limb amputation is one of the major complications. Therefore, intensive care of patients presenting DFU must be considered to avoid amputation. DFU is characterized by impaired tissue regeneration, vasculopathy, neuropathy, and inflammation, which in turn are caused by uncontrolled hyperglycemia and insulin resistance. Indeed, neuropathy is present in 30–50% of diabetic patients, being a good predictor of DFU (Pradhan et al. 2009). Moreover, these ulcers tend to heal very slowly, enabling the appearance of a wide variety of microbial infections and extensive exudate, features that further promote limb amputation (Anisha et al. 2013).

Nevertheless, given the inflammatory and neuropathic environment, as well as the vascular impairment present in the injured skin, application of wound dressings to treat DFU remains a promising approach (Sahana and Rekha 2018). Emerging evidence reports that these dressings must be able to control wound exudate but must also take into account the safeness, comfort, and costs.

The functionalization of breathable alginate hydrogel dressings using curcumin and resveratrol has been proposed for the management of skin wounds through anti-inflammatory and antibacterial effects (Comotto et al. 2019). Although hydrogel dressings stand as the most advantageous ones (Zhang et al. 2019), the use of nanosystems has been receiving increased attention. For instance, insulin-loaded silver nanoparticles have been recently reported to improve wound healing through in situ delivery of insulin and consequent modulation of cytokines, accelerating skin re-epithelialization in diabetic animal models (Kaur et al. 2019). Additionally, the use of hierarchical fibrous membranes with ellipse-shaped nanopores and incorporating dimethyloxalylglycine-loaded mesoporous silica nanoparticles could significantly improve angiogenesis in a diabetic wound bed (Ren et al. 2018).

Several other attempts have been made to use delivery systems targeting diabetic vascular complications, including BDNF gene delivery in neuro-targeted nanoparticles (Lopes et al. 2017), incorporation of glutamate inside hydrogels to accelerate

diabetic wound healing (Thangavel et al. 2017), or nanotherapeutic strategies targeting metabolic disturbances (Sibuyi et al. 2018; Rudnicki et al. 2018).

The fact that polyphenols play benefic roles in inflammation, oxidative stress, angiogenesis, neurodegeneration, and metabolism renders these compounds promising multitask therapeutic molecules in diabetic wound healing. In accordance, studying wound healing in diabetic Wistar rats, our group recently observed that consumption of xanthohumol, a beer-derived polyphenol, was able to reduce the number of microvessels within the wounded area to normal values (Costa et al. 2013). These findings were accompanied by a decrease in blood levels of VEGF and several inflammatory as well as oxidative stress factors. Nevertheless, the fact that most of these agents are not easily soluble in aqueous environments renders them of difficult use. Immobilization of these compounds in adequate biomaterials is a promising tool for therapeutic strategies. Despite the lack of conclusive preclinical animal studies, in vitro experiments using nanosystems immobilizing polyphenols are encouraging.

11.5 Conclusions and Perspectives

Diabetes is a systemic disorder affecting several organs in many distinct ways. The pleiotropic role of polyphenols renders these phytochemicals potential therapeutic agents in the management of diabetic complications. Nonetheless, their chemical properties, bioavailability, digestion, intestinal absorbance, and metabolic rate prevent these agents to exert their broad biological effects. Smart engineering of polyphenol-loaded nanosystems considering the target tissue will definitely overcome major drawbacks associated to these bioactive/therapeutic agents. Strategies for antidiabetic applications are frequently designed to minimize the degradation of polyphenols within the GI tract envisioning a targeted delivery to the intestinal epithelium and increased cellular uptake.

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Chapter 12
Implications of Nanotechnology in Cancer Diagnostics and Therapeutics

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Abstract Cancer is the second leading cause of death worldwide attributed to various factors including changing lifestyle, exposure to chemical carcinogens, environmental pollution, and genetic factors. Such high death rate is partly due to late diagnosis and lack of effective and affordable therapies specifically in developing countries. Although in recent years there have been tremendous progress in improving cancer diagnostics and therapeutic approaches, we are still very far from controlling cancer deaths. Nanotechnology is an upcoming field of science, which deals with nanomaterial at the atomic and molecular level and holds great potential in human health and medicine. The synthesis, characterization, and applications of nanoparticles are among the most important sections of the wide range of nanotechnology areas that have immense potential, particularly in the diagnostic, imaging, and therapeutic domains of cancer research. This chapter reviews the potential of nanomaterials specifically nanoparticles and their applications in developing or improving cancer diagnostics and therapeutic approaches.

Keywords Cancer · Diagnosis · Nanotechnology · Nanoparticles · Therapeutics

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_12

12.1 Introduction

Cancer is a highly complex disease, which develops through multistep process involving uncontrolled cell growth, resistance to cell death, changes in cellular signaling, tissue invasion, angiogenesis, and metastasis (Zou 2005; Reichert and Wenger 2008). Cancer generally starts as localized tumor and can spread to distant sites within the body (metastasis), which makes the treatment difficult. For the detection of cancer (cancer diagnosis), clinical and pathological staging is used that utilizes morphologic diagnostic tools, such as radiological and histopathological examinations. However detailed information on disease progression using these tools is not possible. For treatments, the most common methods are surgical removal, radiation, and chemotherapy, which are also limited due to laborious process, side effects, and chemotherapeutic resistance, respectively (Tannock 1998). To overcome these problems of treatment, apart from surgical and radiation therapies, significant work has been done for developing novel chemotherapeutic agents for targeted cancer treatment in the past several decades (Shewach and Kuchta 2009; Urruticoechea et al. 2010). It includes use of alkylating agents, antimetabolites, natural products, enzymes, hormones, aromatase inhibitors, and receptor-specific monoclonal antibodies for cancer treatment (Shewach and Kuchta 2009). However these are also restricted due to various reasons including nonspecific distribution of anticancer agents, inadequate drug concentrations to the tumor site, cytotoxicity, difficulty in monitoring therapeutic responses, and development of multiple drug resistance. Therefore, current diagnostic and prognostic tools are insufficient to make predictions for successful treatment and patient outcome. There is an urgent need to develop new and innovative technologies that could help to provide specific information such as tumor margins, residual tumor cells, and micrometastases.

To develop a successful anticancer chemotherapeutic agent, desired accumulation of drug at tumor site and minimum inhibitory effect on nearby healthy cells are the key factors for targeted treatment approaches. The notion of targeting has become a significant focus in biological research, and therefore gene and protein targeting are some of the major domains of basic research that answer specific biological queries by finding their specific functions (Capecchi 2005; Marx 2013). In the area of drug discovery and molecular therapeutics, development of small molecules and monoclonal antibodies including imatinib, trastuzumab, bevacizumab, and rituximab has revolutionized the therapeutic approach of cancer (Coiffier et al. 2002; O'Brien et al. 2003; Yang et al. 2003; Romond et al. 2005). Current researches are focused on combining conventional therapeutic drugs with synthetic or natural agents as carriers for targeted delivery that could be exploited further for improving cancer prevention, detection, and treatment strategies (Bayat Mokhtari et al. 2017). In this context, several ligand-targeted therapeutic approaches such as drug-immune conjugates, immunotoxins, and radioimmunotherapeutics are being developed to overcome the side effects of conventional chemotherapeutic drugs, thereby providing additional tools in cancer therapy (Srinivasarao and Low 2017). Although these conjugated agents have shown promising efficacy compared with conventional

chemotherapy drugs, efficient delivery of these conjugates to targeted site still remains a major challenge, limiting this approach.

Nanotechnology is an emerging area of science, which involves the creation and manipulation of materials at nanoscale levels (nanoparticles) to generate products that exhibit novel properties. Since nanoparticles can be designed with distinctive shapes, framework, sizes, and surface chemistries, it can be used to develop novel agents in a wide range of biological applications. Their unique properties and behavior in biological system also make it exciting and integrative approaches for studying fundamental biological questions (Salata 2004). The use of nanotechnology in cancer is emerging as a new field of interdisciplinary research cutting across the disciplines of chemistry, engineering, biology, and medicine and is expected to improve our current detection, diagnosis, and treatment methods (Ferrari 2005). Specifically in cancer therapeutics, the concept of developing more effective cancer treatments by engineering matter at the nanoscale alone or in combination with chemotherapeutic drugs provides a great opportunity for cancer researchers to efficiently target cancer cells without damaging the normal cells (Brigger et al. 2002).

12.2 Nanoparticles

Nanotechnology is a new branch of science with a variety of applications from energy production to industrial production processes to biomedical applications (Salata 2004; O'Farrell et al. 2006; Rudramurthy and Swamy 2018). To define nanotechnology, it is the approach of manufacturing nanomaterials at the atomic and molecular level, where nanomaterials such as nanoparticles, nanotubes, nanosheets, and nanorods have a size ranging from 1 to 1000 nm in at least one dimension. The synthesis, characterization, and applications of nanoparticles (NPs) are among the most important sections of the wide range of nanotechnology. Nanoparticle may be defined as the colloidal system generally submicronic (1–100 nm range) and made up of a variety of materials including polymers (i.e., biodegradable nanoparticles), inorganic materials (i.e., metal nanoparticles), lipids (i.e., liposomes, solid-liquid nanoparticles), and biological materials (i.e., viral and albumin nanoparticles). These nanoparticles can be engineered to possess a unique composition and functionality, and as per the requirements, it can also be customized as nano-spheres or nano-capsules.

Nanoparticles either are made from single material referred as "simple nanoparticle" or can be a composite (core/shell) nanoparticle, which is made up of two or more materials. The core/shell nanoparticle is composed of core (inner material) and shell (outer layer material). The shell is made up of various combinations of organic and inorganic materials, and their choice of material is generally dependent on its application and use (Ghosh Chaudhuri and Paria 2012). Generally nanoparticles are synthesized by various methods, which can be classified mainly as top-down and bottom-up approaches (Wang and Xia 2004). In top-down approach, the

desired shape and order of nanoparticles are achieved through cutting and milling using microfabrication method. It includes lithographic techniques such as UV, electron or ion beam, scanning probe, optical near-field laser-beam processing, and mechanical techniques such as machining, grinding, and polishing. On the other hand, "bottom-up" approaches utilize the chemical properties of the molecules for self-assembly into certain useful conformation. It includes chemical synthesis, chemical vapor deposition, laser-induced assembly, self-assembly, colloidal aggregation, film deposition, and growth. Both of these methods have its limitations and advantages over one another and therefore are used according to the user's requirements either alone or in combination (Wang and Xia 2004). For example, to synthesize small-sized particles, bottom-up approach has advantage of being low cost, precise, regulatable process with minimum energy loss. However, for the synthesis of composite nanoparticles, both approaches could be useful, such as core nanoparticles are synthesized with more precision using top-down approach and shell is synthesized using bottom-up approach because it provides a uniform coating of the shell during particle formation. Therefore combination of the two approaches could also be utilized for specific applications.

With increasing advancements in the synthesis techniques, these nanoparticles can be synthesized following various chemical, physical, and biological methods (Iravani et al. 2014). In addition, these NPs can be synthesized in various shapes such as symmetrical (spherical), cube, prism, hexagon, octahedron, disk, wire, tube, and rod. Changes in the shape also contribute in altering the physical and chemical properties of nanoparticles (Albanese et al. 2012). For example, during the synthesis of magnetic nanocrystals, properties such as blocking temperature and magnetization depend on size of the particles, while its reactivity is dependent on shape and surface properties (Song and Zhang 2004; Xiao et al. 2007). These magnetic nanocrystals with varying shapes possess tremendous potential in improving our basic knowledge of magnetism, which can be utilized further in technological applications such as high-density information storage. Similarly, other properties of nanoparticles such as catalytic activity, selectivity, optical and electrical properties, and melting point are also dependent on particle shape (Chen et al. 2005; Stuart et al. 2005; Millstone et al. 2009). These synthesized nanoparticles are further characterized to confirm their properties including size, shell thickness, elemental and surface analysis, optical properties, and thermal stability, among others, using various characterization techniques. The most frequently used techniques for their characterization are dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), thermal gravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS), photoluminescence (PL), and UV-visible spectroscopy.

Nanoparticles show promising result in biological applications and offer many unique properties that enhance or confer advantages over current techniques in biological and biomedical research. In the following sections, we will review some of the applications of nanoparticles specifically in cancer imaging and therapeutics.

12.3 Role of Nanotechnology in Cancer Diagnosis

To detect cancer, tumor imaging is an important tool in clinical oncology, and conventional techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) provide crucial information on anatomic location, extent, and size of the tumor at various levels of spatial resolution and contrast. However, CT and MRI have limited sensitivity and are unable to provide specific and functional information of tumor, which is one of the major obstacles for reliable early diagnosis and subsequently in the monitoring of treatment responses. In this context, molecular imaging is relatively a better choice, which focuses on visualizing or imaging biological events and processes in living systems, including patients (Atri 2006). One of the most commonly used molecular imaging methods is positron emission tomography (PET), which is based on the metabolic feature of tumor in which tumor cells utilizes more glucose due to their high dependency on glycolysis (Gambhir 2002). In this method, a labeled analogue of glucose, i.e., fluorodeoxyglucose (FDG), is used as PET imaging probe, which accumulates mainly in tumor cells and allows the detection of specific tumors with high sensitivity. However, such methods may not be suitable for certain tumors showing low glucose uptake. Other highly sensitive and noninvasive methods of tumor imaging are available which include single-photon emission tomography and optical imaging including fluorescence-mediated tomography and near-infrared fluorescence (NIRF) reflectance imaging (Gambhir 2002; Allen and Cullis 2004; Kjaer 2006; Weissleder 2006; Iagaru et al. 2007;). It is well recognized that early cancer detection and effective therapy improve patient survival; the use of nanoparticles as imaging contrast agents also makes it a good candidate for targeted tumor imaging, as well as delivery of therapeutic agents due to their multifunctional properties. Therefore in comparison with radioactive probes (F-labeled FDG) in PET imaging, nanoparticles have both greater surface areas and more functional groups that can be linked with multiple diagnostic and therapeutic agents. Similarly, biomarker molecules, which are specifically produced by cancer cells, can be coupled with imaging probes guided by ligands that can recognize and interact with target molecules and allow targeted detection of cancer and monitoring of chemotherapeutic treatment. Below are some of the important examples of nanoparticle-based approaches that have potential to improve our current tumor imaging techniques.

12.3.1 Nanostructure-Based Biosensor

Biosensor is primarily a biological recognition element, which can detect and quantify the presence of biological molecule in the solution along with its activity or concentration through biochemical reaction (Jianrong et al. 2004; Singh et al. 2020). These biochemical reactions could be based on antigen-antibody interaction, DNA hybridization, or binding of capture ligand to the cell surface epitope (Begent et al.

1996; Vo-Dinh et al. 2001; Wu et al. 2011). It is followed by converting the biochemical reaction result into a quantifiable signal using transducer as a part of the detection device. The transduction mechanisms rely on light, magnetic, or electronic effects (Vo-Dinh et al. 2001). For example, benzo[a]pyrene (BaP) is a common environmental pollutant as well as a component of tobacco smoke and is a known human carcinogen. It can be detected by biosensors utilizing antibody-based system for the detection of BaP tetrol in the body, which indicates exposure to BaP. Early detection of exposure to BaP has remarkable implications in the detection and treatment of, in particular. Lung cancer, which is a leading cause of cancer death worldwide (Wu et al. 2011). These biosensors can be combined with nanofibers or nanoparticles to create nanobiosensors, which can improve the efficiency of detection methods. For example, the rate of angiogenesis (formation of new blood vessels) is high in tumor tissue and is related to proliferation and metastasis of cancer cells (Kwon et al. 2010). Vascular endothelial growth factor (VEGF) is a potent angiogenic biomarker and is crucial for angiogenic process and thus considered as an attractive target for early cancer diagnosis and subsequent therapy (Kwon et al. 2010). Current methods for the detection VEGF utilize field-effect transistor biosensors with VEGF receptors to detect VEGF molecules, and quantification of this ligand-receptor complex is done by ELISA (enzyme-linked immunosorbent assay). However to further improve the sensitivity of this system, modified biosensor is designed which is a combination of conducting polymer (polypyrrole) nanotubes with aptamers (nano-sized molecules with high degree of sensitivity and specificity for target molecules), bonded to the nanotubes (Kwon et al. 2010). In this system two different sizes of carboxylated polypyrrole carbon nanotubes (CPNT) were synthesized (CPNT 1, 190–220 nm; CPNT 2, 100–130 nm) and attached to biosensors and examined for lower levels of VEGF for detection. Although both CPNTs displayed higher sensitivity in detecting VEGF, the CPNT 2 showed twofold higher sensitivity than CPNT 1, indicating the significance of size in this detection method. The presence of aptamer on CPNT 2 produced superior sensitivity compared with non-aptamer-based biosensors and the larger size of CPNT 1. In addition, rapid real-time detection adds another favorable dimension to this system, as the biological events detected can be converted directly into a measurable signal without labeling of samples. In addition, detection by fluorescence and luminescence methods has also been introduced to improve the sensitivity and detection limit (Wang et al. 2005b). Since VEGF concentrations are very low at the early stages of metastasis, such nano-based systems could have enormous implications for the early detection of several metastatic cancers.

Similarly telomerase is an enzyme which maintains the telomere length and is highly expressed in tumor cells. Therefore telomerase is considered as potential biomarker for early cancer detection, as well as monitoring the efficacy of chemotherapeutic interventions (Zheng and Li 2010). Since telomerase is located in trace amounts in the nucleus, its detection requires ultrasensitive single-cell analysis method in living cells. Nanofiber biosensors conjugated with anti-telomerase antibody can directly bind to telomerase and detect low levels of telomerase in the nucleus. Due to its small size, these nanofiber biosensors are also capable of

penetrating nucleus and able to specifically detect telomerase only in the nucleus, making telomerase detection by nano-systems a superior detection technique.

12.3.2 Quantum Dot Nanoparticles

Semiconductor quantum dots (QDs) are one of the other most promising nanoparticles for improving the sensitivity of molecular imaging and quantitative cellular analysis by one to two orders of magnitude. The QDs are nanometer-scale, light-emitting particles with unique optical and electronic properties such as variable size light emission, enhanced signal brightness, greater stability of the fluorescent signal, and wide range of excitability in the fluorescent spectrum. One of the first examples of QDs in tumor imaging was the use of bioconjugated prostate membrane antigen-targeted QDs, which can simultaneously target and image prostate tumors in living animal models (Chan and Nie 1998). These QD conjugates consist of amphiphilic copolymer layer and polyethylene glycol (PEG) molecules, which make them stable and biocompatible. In addition, these conjugates are also able to produce brighter signal when used in in vivo systems (Gao et al. 2004). Based on their differential spectral properties, it can be used for imaging and co-localization studies for simultaneous tracking of multiple markers with improved sensitivity and specificity in cancer diagnostics. In addition, these QDs can be modified to produce near-infrared fluorescence (NIRF) signals in which light penetrates more deeply in tissues than visible fluorescence range and allows the detection of signals from thick tissues in animals. One of the major advantages of using NIRF QDs is that their emission range is beyond the autofluorescence spectral range produced by blood or tissues, resulting in high signal-to-background ratio during imaging (Soltesz et al. 2005; Cai et al. 2006). Sensitive real-time detection of tissue distribution of targeted QDs is also possible using the NIRF optical imaging system after systemic delivery. These NIRF QD system has been successfully used for real-time imaging of sentinel lymph nodes within large animals (Weissleder 2006). Therefore, these QDs hold great promises as optical imaging nanoprobes for evaluating the specificity of tumor-targeting ligands. Since these QDs also contains some of the potential toxic components such as cadmium, further studies are also required to investigate their side effects, toxicity, and feasibility for future clinical applications.

12.3.3 Magnetic Iron Oxide Nanoparticles

Superparamagnetic iron oxide (SPIO) or iron oxide (IO) nanoparticles are other attractive tools, which can be used as the precursor for the development of a target-specific MRI contrast agent. Different modifications of IO nanoparticles have been used in clinical settings and have proven to be safe for human use (Hamm et al. 1994; Bulte and Kraitchman 2004b; Maier-Hauff et al. 2007). Some recent studies

have demonstrated that various cell lines can internalize IO nanoparticles that allows for magnetic labeling of the targeted cells (Moore et al. 1997; Kircher et al. 2003). In case of SPIOs, due to their paramagnetic properties and forming a stable colloidal suspension, they have significant biomedical applications, especially in tumor imaging, in which they induce stronger T2 and contrast and can be utilized as MRI contrast agent (Wang et al. 2013). With the magnetic force, it can be directed to a specific site in the body making them useful for targeted diagnosis and/or therapy. Therefore, SPIO can be used in a variety of applications, such as stem cell tracking (Bulte and Kraitchman 2004a; Daldrup-Link et al. 2005), magnetic separation (Xu et al. 2011), hyperthermia therapy (Kobayashi 2011), and anticancer drug delivery (McBain et al. 2008), which offer higher intracellular concentration. MR contrast imaging with SPIOs for liver tumor and lymph node metastasis is one of the known examples of clinical applications of SPIOs (Wang et al. 2001). These SPIO has been shown to be capable of the differentiation of the lesions that are as small as only 2–3 mm (Corot et al. 2006). In addition, these ultrasmall supermagnetic iron oxides (USPIOs) are also very effective in the imaging of the metastasis of the lymph nodes with only 5–10 mm of diameter (Harisinghani et al. 2003). In addition, IO nanoparticles have a long blood-retention time and are generally biodegradable and therefore considered to have low toxicity, making it a very attractive area in noninvasive diagnosis of cancer (Sjögren et al. 1997; Weissleder 2006).

12.3.4 Other Examples

In case of colorectal cancer, colorectal lining is examined with the aid of a fiber-optic camera (colonoscopy), which allows the detection of small growths in the colorectal tissue. To improve the accuracy of colonoscopy and detection at the early stages, gold nanoparticle-based approach has been developed that identifies and binds to cancer cells (Lin et al. 2011). When light from the colonoscope is thrown over the colorectal tissue, bound nanoparticles outshine and are utilized further for specific removal of the colon tissue/cells from the colon lining. Clinical trials are underway to test the safety of this approach in humans (Lin et al. 2011; Kim et al. 2016).

Since patient samples such as blood/serum can easily be obtained, detection of tumor-specific biomarkers using conjugated nanoparticles with these samples in immunoassays could greatly enhance their sensitivity (Arruebo et al. 2009). For example, the bio-barcode assay was designed as a sandwich immunoassay which captures the target proteins through magnetic nanoparticles (MMPs) attached with protein-specific monoclonal antibodies. The MMP-protein hybrid structures are then combined with gold nanoparticle (Au-NP) probes, which carry DNA barcodes. Target protein-specific DNA barcodes are released into solution and can be detected in femto- or picomolar range using scanometric assay (Huang et al. 2017).

12.4 Nanotechnology as Drug Delivery System

In recent times, nanotechnology and nanoparticles have generated great interest in cancer therapeutics due to their ability to offer improved and targeted drug delivery systems to overcome the limitations in conventional chemotherapy (Yoo et al. 2010; Koo et al. 2011; Prasad et al. 2017). The limitations of chemotherapy include inefficient drug delivery at the target site due to physical and biochemical barriers and drug resistance at the tumor level due to cellular and noncellular mechanisms, which limit the drug effect and result in reoccurrence and high mortality rate. Poorly developed vascularized tumor regions and tumor microenvironment are the major mechanisms that may hinder the drug access to the tumor tissue and can reduce the drug effect. Similarly, increased interstitial pressure and decreased microvascular pressure may also retard extravasation of drug molecules (Krishna and Mayer 2000). Therefore to overcome these limitations, developing nanoscale delivery carriers for anticancer drug molecules with nanoparticles could address the pharmacokinetic drawbacks of conventional drug formulations and could target the drug to cancer cells with controlled rate and efficacy (Blanco et al. 2015). Since nanoparticles can be prepared from different materials, including polymers, metals, and ceramics, they can be modified in diverse shapes and sizes with distinct properties and can be used as a vehicle for efficient drug delivery system. Various types of nanoparticles in combination with anticancer drugs are under development to enhance drug efficacy including liposomes, solid lipid nanoparticles, dendrimers, and solid metal-containing NPs (Hu et al. 2010; Ventola 2017). An updated list of nanoparticle-based formulations that are either FDA approved or in the clinical trials, including cancer, has been summarized in the review by Bobo et al. (2016). Some of the major forms of nanoparticles that have potential in cancer therapeutics are discussed below (Table 12.1).

12.4.1 Liposomes

Liposomes are nm to mm small-sized molecules, artificially developed from cholesterol and amphiphilic phospholipids, and can be designed in various shapes. These liposomes are very versatile tools in nanomedicine as they are biocompatible and biodegradable, and due to their amphiphilic nature, they can be combined with both hydrophobic and hydrophilic compounds. Liposome-conjugated drug delivery system is one of the most established approaches in cancer treatment with several liposomal-conjugated anticancer drug formulations approved by FDA (Ventola 2017). The examples include liposome combination with drugs like daunorubicin (DaunoXome) for Kaposi's sarcoma and doxorubicin (Doxil/Caelyx) monotherapy for Kaposi's sarcoma and ovarian cancer, as well as in combined therapy with bortezomib for multiple myeloma, cytarabine (DepoCyt) for lymphatic meningitis, vincristine (Marqibo) for acute lymphoblastic leukemia, and irinotecan (Onivyde), for

Table 12.1 Some of the major nanoparticle-based modifications in cancer therapeutics

Types of nanoparticles	Drug	Formulation	Development stage	Cancer type
Liposomes	Doxil	PEG-liposome dox	FDA approved	Kaposi's sarcoma, ovarian cancer
	DaunoXome	Liposomal daunorubicin	FDA approved	Kaposi's sarcoma
	Marqibo (Onco TCS)	Liposomal vincristine	FDA approved	Acute lymphoblastic leukemia
	Onivyde (Merrimack)	Liposomal irinotecan	FDA approved	Pancreatic cancer
Albumin-based particles	Abraxane	Albumin/ paclitaxel	FDA approved	Breast cancer, NSCLC, pancreatic cancer
Polymeric micelles	SP1049C	Pluronic micelle/ dox	Clinical trails	Gastric cancer
	Genexol-PM	PEG-PLA micelle/paclitaxel	Marketed in Europe and Korea	Breast cancer
	NK 911	PEG-ASP micelle/dox	Clinical trails	Pancreatic cancer
Polymer-based particles	XYOTAX	PG/paclitaxel	Clinical trails	Lung cancer
	IT-101	CD polymer/ CPT	Clinical trails	Solid tumors
	CT-2106	PG/CPT	Clinical trails	Ovarian cancer

pancreatic cancer (Bobo et al. 2016). Similarly, these liposomal-conjugated drugs can be targeted to specific cell type with specific receptor such as folate-receptor-targeted liposomes and have been used to effectively deliver doxorubicin in vivo and can also bypass MDR in tumor cells (Immordino et al. 2006).

12.4.2 Dendrimers

Dendrimers are branched polymeric macromolecules, which can be altered in different shapes and sizes and therefore serve as useful nanocarriers for drug delivery in cancer. It is mainly comprised of a central core, repetitive branching units, and terminal groups that provide flexibility to alter their surface functions based on the requirements. Their structures can be regulated by altering the number of repeated branching making them a suitable choice for attaching to oligonucleotide or drug molecule to a desired shape such as globular structure (Lee et al. 2005; Immordino et al. 2006). Oligonucleotides or chemotherapeutic drugs can be attached to their surface or encapsulated in their internal cavities through hydrophobic or electrostatic interactions. Similarly, these agents can also be covalently attached to terminal functional groups of the dendrimers. Different types of polymeric dendrimers

have been developed for conjugation to target molecules including poly(amidoamine) (PAMAM), poly(propylene imine) (PPI), poly-L-lysine (PLL), carbosilane, and triazine dendrimers (Choi et al. 1999; Kim et al. 2007; Perez et al. 2009; Ionov et al. 2012). Among these dendrimers PAMAM, PPI, and PLL dendrimers are commonly used as chemotherapeutic drug conjugates. These dendrimers are useful for conjugating the anticancer drugs specially which are hydrophobic in nature and difficult to administer such as paclitaxel, camptothecin, methotrexate, 5-fluorouracil, and DOX free base. Due to cytotoxic nature of these drugs, the use of dendrimers as carrier would provide additional advantage of minimizing side effects. For example, DOX-PAMAM dendrimer conjugates have shown promising results in reducing tumor burden and increased accumulation of drug in lung metastasis (Zhong et al. 2016). Similarly in breast cancer models, encapsulating docetaxel (DTX) modified with trastuzumab (TZ) which are the example of combinatorial therapy, the uptake TZ-DTX dendrimers with DTX dendrimers shown to be increased with high efficacy (Kulhari et al. 2016). PPI dendrimers conjugated with drugs like paclitaxel and melphalan formulations promoted higher tumor inhibition, as well as survival rates in ovarian carcinoma and breast cancer models, respectively (Jain et al. 2015a, b).

12.4.3 Polymeric Micelles

Polymeric micelles are amphiphilic block copolymers synthesized as core/shell structures and useful for drug delivery agents as it can improve the solubility of hydrophobic drugs, sustain release, and protect them from enzymatic degradation (Lavasanifar et al. 2002). Generally, polyethylene glycol is used as hydrophilic block to synthesize di-block, triblock, and graft copolymers of micelles. Other hydrophilic block-forming polymers include poly(N-vinylpyrrolidone) (PVP), poly(N-isopropylacrylamide) (pNIPAAm), and chitosan. Similarly, various polymer blocks are utilized to form micellar core, such as poly(propylene oxide) (PPO), poly(L-lactide) (PLA), poly-ε-caprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(β-amino esters), polyamino acids including poly(L-histidine) (pHis), poly(L-aspartic acid) (pAsp), and lipids such as dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidylethanolamine (DSPE) (Nakanishi et al. 2001). These are used in conjugation with chemotherapeutic drugs to improve their efficacy (Bae et al. 2005). One of the first examples of polymeric micelle formulation with chemotherapeutic drugs is Genexol-PM (PEG-poly(D,L-lactide)-paclitaxel), which is very effective and approved for breast cancer treatment (Kim 2004). Similarly, several PEG-based micelle formulations are under clinical trials such as doxorubicin-loaded polymeric micelle and have shown promising results for solid tumors (Torchilin et al. 2003; Mohanty et al. 2010). Other modifications such as antitumor antibody-conjugated polymeric micelles (immunomicelles), encapsulating the water-insoluble drug Taxol, have been tested for their efficacy in vitro and shown that these conjugates effectively recognize cancer cells (Wang et al. 2005a). For example, in pancreatic cancer, curcumin-loaded methoxy PEG/PCL

copolymeric micelles showed better efficacy than native drug (Mohanty et al. 2010). However, in some cases, safety of these formulations is also a concern for their use in treatment approaches (Rollerova et al. 2015).

12.4.4 Carbon Nanotubes

One of the latest systems in nanoparticles is the carbon nanotube which is carbon alloform framed in cylindrical network, composed of benzene rings with wider applicability to be used as sensors for detecting biomolecules including DNA and protein. These are classified as single-walled carbon nanotubes (SWNCTs) and multiwalled carbon nanotubes (MWCNTs), based on number of sheets in concentric cylinders. Since carbon nanotubes have highly hydrophobic hollow interior, it can be used to conjugate water-insoluble drugs. In addition, their large surface area is useful for outer surface functionalization and can be modified for a particular cancer receptor as well as contrast agents (Lamanna et al. 2012). It can be used as diagnostic devices for detecting various serum proteins and also can serve as carriers to deliver cancer drugs, vaccine, or protein (Bachilo 2002). These (SWNCTs) are being explored as biosensors and drug transporters by utilizing their structural, mechanical, electrical, and optical properties (Nune et al. 2009). These SWNCTs have high optical absorbance in the near-infrared range, which can be used to generate heat by laser irradiation, and are useful for destroying cancer cells that selectively internalized with nanotubes. Since near-infrared (NIR) fluorescence is biologically transparent region (700–1300 nm) where autofluorescence, absorption, and scattering by blood and tissue could be minimized, NIR properties of SWNCTS could be used for biomedical imaging. Surface-functionalized multiwalled carbon nanotubes have also been used successfully for bio-imaging, and cells can internalize it more efficiently than the drug alone.

12.5 Mechanism of Nanoparticles in Targeted Drug Delivery

Nanoparticles exhibit several features including physiochemical properties, solubility, degradation, and clearance which make them suitable candidates for developing diagnostics as well as therapeutics, commonly called as “theranostics.” Their small size is one of the important features, which allows them to travel through the bloodstream and subsequent delivery of the nanocarriers to tumor tissue. Smaller nanoparticles have more advantage as it easily accumulates in the leaky blood vessels of tumors and can also escape to surrounding tissues compared with large-sized particles ((Bregoli et al. 2016). Along with size, the shape of the nanoparticles also impacts fluid dynamics and thus influences the nanocarrier uptake. Spherical nanoparticles are most commonly used nanocarriers as it can be easily synthesized and characterized (Truong et al. 2014). Similarly, electrostatic properties of the

nanoparticle such as charge also affect their stability and distribution in circulation and targeted tissue. For example, positively charged nanoparticles accumulate more in tumor vessels, and changing to neutral charge after extravasation allows their faster diffusion to the tumor tissue (Stylianopoulos et al. 2010). These properties of nanoparticles are being used to modify or improve the drug molecule or ligand effectiveness by increasing their stability, solubility, and bioavailability. Delivery of these nanoparticles with anticancer drugs to circulation or targeted tissue is generally achieved by the following major mechanisms.

12.5.1 Passive Targeting

Passive targeting refers to the passive accumulation of nanocarriers or its drug conjugates at the targeted site due to the unique properties of tumor vasculature, such as the enhanced permeability and retention (EPR) effect and the tumor microenvironment. With this, targeting nanoparticles passively accumulates in the leaky blood vasculature exhibited by tumors without any surface modifications (Albanese et al. 2012; Wicki et al. 2015; Bregoli et al. 2016). Passive targeting uses the anatomic and functional differences between normal and tumor tissues and therefore can effectively increase the bioavailability and efficacy of the drug at the localized site. The anatomical differences of the tumor tissues include large gaps between angiogenic vessels and epithelial cells, while functional changes include elevated levels of various molecules such as bradykinins, nitric oxides, and growth factors. Pathophysiological features of tumor vasculature include poor lymphatic drainage which can induce the EPR effect and enable macromolecules, including nanoparticles, to escape through these gaps into extravascular spaces for accumulation inside tumor tissues (Maeda et al. 2000). Since tumor cells require increased oxygen supply and nutrients to meet their energy demands, these cells are metabolically reprogrammed to compensate these requirements and therefore show altered tumor microenvironment, which could be utilized in passive targeting of drug. These changes in tumor tissue or microenvironments can enhance the delivery of drug when it is conjugated with nanocarriers. For example, tumor cells have high glycolysis rate, which results in more lactate formation leading to an acidic environment in the surroundings of tumor cells. In this scenario, chemotherapeutic drugs, which work at low pH, can be encapsulated with pH-sensitive liposomes. These liposomes are stable at physiologic pH of 7.4 but degrade at low pH, which will allow the release of drug in target tissues for its activity in suitable acidic environment of tumor cell resulting in an improved efficacy of drug. Several therapeutic drugs including peptides, cisplatin, doxorubicin, ovalbumin (OVA), and antigen base molecules have been tested for their effective delivery to the tumor tissues with these liposome conjugates (Paliwal et al. 2014). Similarly albumin-bound form of doxorubicin incorporating an enzyme matrix-metalloproteinase-2-specific octapeptide sequence has been used in conjugation in cancer cells (Mansour et al. 2003). These metalloproteinases act efficiently and specifically on albumin and

doxorubicin and cleave the conjugation to release the doxorubicin. Overall, passive targeting exploits the cancer cell properties for the selective and efficient drug delivery along with nanocarriers.

12.5.2 Active Targeting

Since passive targeting utilizes EPR effect and tumor microenvironments to promote some tumor-selective delivery of nanoparticles to tumor tissues, it has a major drawback that it cannot differentiate between normal and tumor tissue efficiently and therefore could also affect healthy cells/tissue leading to side effects. Therefore an alternate approach is required with most suitable targeting agent or agents, which can selectively and successfully transport nanoparticle systems to cancer tissue to avoid any kind of toxicity in the process. These targeting agents could be ligands or antibody from cancer cells which has capability to bind to the tumor cell surface with an extremely strong affinity to trigger receptor endocytosis. With these interactions, the therapeutic agents will then be delivered into the tumor-specific regions with minimal cytotoxicity (Bazak et al. 2015). Active targeting involves the attachment of a homing moiety, ligand-receptor, antigen-monoclonal antibody, or other molecular recognition to confer more specificity to the delivery system and reduces the unwanted nonspecific interactions and localization of the drug in peripheral tissues (Brigger et al. 2002; Huynh et al. 2009). Some of the major criteria for selecting these agents are as follows: (1) these antigen or receptor should be uniquely expressed on cancer cells, (2) these should be expressed homogeneously on all targeted tumor cells, and (3) these antigens or receptors should not shed into circulation. These parameters allow the efficient internalization of targeted conjugates after binding to target cells which involves receptor-mediated endocytosis. Folate receptor targeting is one of such examples where folate receptor is highly expressed in several cancers and folate-targeted conjugates (i.e., doxorubicin-conjugated poly(D,L-lactic-co-glycolic acid (PLGA)-polyethylene glycol (PEG) particles) can be actively targeted and efficiently internalized by cancer cells (Sah et al. 2013). Similarly, antibody conjugates against specific growth factors could also be used for active targeting of chemotherapeutic agents in cancer cells. For example, F5 antibody binds to ErbB2 growth factor, which is overexpressed in human breast cancer and also in several other adenocarcinomas (Nielsen et al. 2002). This F5 antibody is used in conjugation with liposome-doxorubicin-PEG system (F5-coupled Doxil) and shown to be effective in reducing tumor volume as compared with free Doxil in a cancer mouse model (Nielsen et al. 2002). These ligands targeting cell surface receptors have the advantages of lower molecular weight and lower immunogenicity than antibodies. However some ligands such as folate are also present in food and high concentrations in human body; it might compete with the nanoparticle-conjugated ligand for binding to receptor and therefore may reduce the intracellular concentration of delivered drug.

12.5.3 Limitations of NPs in Cancer Therapy

Although nanotechnology and nanoparticles offer specificity of conventional therapies, there are also many challenges associated with nanotechnology. For example, some of the cancer cell types develop drug resistance over drug treatment causing the drug released by nanoparticles ineffective. Likewise, conjugation of nanoparticles with drug might change the solubility, stability, and pharmacokinetics of the carried drugs and could possibly reduce the effectiveness. Other limitations include shelf life, toxicity, aggregation, and leakage of material used to make nanoparticles. Some materials which are used to make nanoparticles have low toxicity but have a very low half-life and stability in circulation and therefore do not circulate in tissue for sustained drug delivery (e.g., poly(lactic-co-glycolic acid) (PLGA)). Although some materials such as carbon nanotubes and quantum dots have greater stability in the body (weeks or months), it may be potentially toxic, hence limiting their use for treatment repeatedly (Jain et al. 2015a). To overcome such problems, new materials are being exploited for generating targeted nanoparticles such as silicon or silica; however their use on cancer patients has been restricted due to the lack of knowledge about their long-term effect on human health. Similarly, a majority of the nanoparticle-based studies have been reported in cell culture models, and investigations in animal models also lack detailed information on their distribution, metabolism, and nanoparticle clearance. Despite extensive research efforts to develop new targeted nanoparticles, only few of them (i.e., Abraxane, Doxil, and Myocet) have been approved by FDA. Ongoing researches in combining nanoparticles with other approaches such as chemotherapeutics, photodynamic therapy (PDT), radiotherapy, and gene therapy are being explored, which may provide alternatives to the limitations of current nanoparticles for targeted delivery and effective cytotoxicity of cancer cells.

12.6 Conclusion

Nanoparticle-based approaches are effective and have potential to improve current diagnosis and treatment approaches for human health and diseases. Multifunctional nanoparticles can be designed in combination with various agents to overcome limitations of conventional chemotherapeutics including nonspecific drug effects. Improving the specificity of the nanocarriers, optimizing the loading of chemotherapeutic drug, and regulating the release of these nanocarriers are of great importance to significantly enhance the drug efficiency for cancer treatment (Fig. 12.1). The use of different variety of nanoparticles would be useful for enhancing the solubility of hydrophobic drugs and stability in circulation, maximizing uptake in cancer cells, preventing undesirable side effects, and improving intracellular penetration for specific cancer targeting. In addition, real-time monitoring of these nanoparticles in detection methods makes them a good candidate for improving the precision

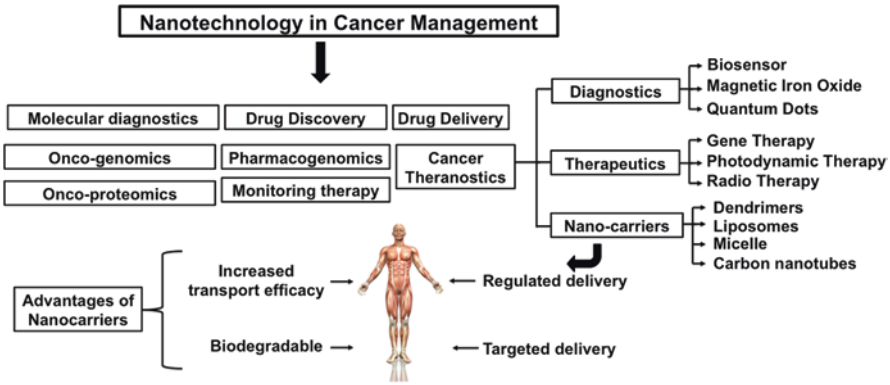

Fig. 12.1 Major highlights of nanotechnology in cancer management including their usage in diagnostics and therapeutics

in cancer diagnosis. Although the use of nanoparticles requires careful investigation in in vivo models of disease and in human subjects, it holds a great promise in future cancer theranostics.

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Chapter 13
Theranostic Nanocarriers in Cancer: Dual Capabilities on a Single Platform

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Abstract The evolution of nanotechnology has ushered in tremendous advances in the field of medicine. Nanocarriers such as liposomes, polymeric and lipidic nanoparticles, micelles, dendrimers, etc., are now used as targeted drug delivery systems that are capable of delivering the therapeutic agent to a specific site in human body. Therapeutic agents such as antimicrobials, anti-HIV, anti-Alzheimer's, immunosuppressants, peptides, genes, as well as anti-cancer drugs, have been successfully delivered using nanocarriers for the treatment of various diseases. Early diagnosis of the disease is the key for successful management of disease. In the case of cancer, early diagnosis is critical as it can have an impact on the treatment measures and the outcomes of treatment, which affect the quality of life of the patient. The diagnostic tools used nowadays consist of various techniques such as magnetic resonance imaging, optical imaging, computed tomography, etc. These tools require diagnostic agents comprising fluorescent dyes, radioactive elements and contrast agents. These diagnostic agents are frequently administered to the patients along with chemotherapeutic agents for the detection and treatment of cancer or to monitor treatment efficiency, which generally causes toxicity in the patients. This drawback initiated a quest for development of a novel platform that is capable of simultaneous diagnosis and therapy, thereby reducing toxicity in the treatment of cancer. The outcomes of this quest are theranostic nanocarriers, which are the next generation of nanocarriers. They combine the therapeutic moieties as well as the diagnostic agents on a single platform and consist of liposomes, polymeric or metallic nanoparticles, micelles, and dendrimers as carriers. Ideally, theranostic carriers must be capable of incorporating both the diagnostic and therapeutic agents in

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_13

potent quantities; they must deliver the therapeutic agent to the site of action and should be non-toxic. In this chapter, we discuss the current scenario of theranostic nanocarriers, their methods of synthesis, the diagnostic agents and therapeutic agents used in theranostic nanocarriers and their applications in the management of cancer.

Keywords Theranostic · Nanocarriers · Cancer · Targeting

13.1 Introduction

The term "Theranostic" is a combination of two terms, therapeutic and diagnostic, and involves a system having a dual capability of therapy and diagnosis. The theranostic nanocarriers as represented in Fig. 13.1 are systems possessing dual ability of targeted drug delivery and diagnosis and may consist of liposomes, polymeric or metallic nanoparticles, dendrimers, nanocages, etc. In the last decade, the concept of "theranostics" came into existence owing to advancements in the treatment of diseases like cancer that require accurate diagnosis and planned strategies of treatment. The main purpose is to protect the life of the patient from the toxicity arising from the repetitive use of diagnostic agents, as well as anti-proliferative drugs, which themselves are toxic in nature.

13.2 Diagnostic Imaging Techniques

Diagnostic imaging aids us to observe biological events at cellular and subcellular levels in a living organism or human. The imaging techniques help us to understand the stages of diseases such as cancer and their progression. These techniques include optical imaging, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT), single photon emission computed tomography (SPECT) and positron emission tomography (PET). All these techniques require molecular imaging agents or contrast agents for enhancement of the images (Janib and Moses 2010).

13.2.1 Optical Imaging

This technique is relatively inexpensive. It involves the detection of low-energy photons ranging from visible to near infrared spectra using contrast agents or dyes. Its main advantage is the avoidance of the ionising radiation that is used in other techniques. However, its drawbacks include: poor tissue penetration, tissue autofluorescence (257–280 nm) and the absorption of light by proteins such as

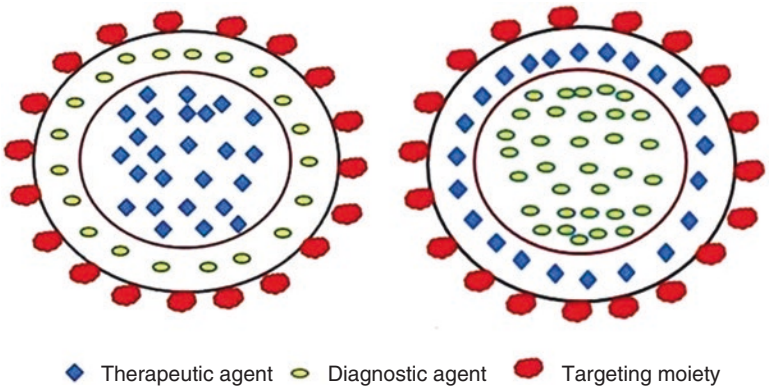

Fig. 13.1 Schematic representation of a theranostic nanocarrier

haemoglobin (560 nm). This technique is also highly vulnerable to background noise caused by scattering of photons by the biological tissue (395–600 nm). However, the near-infrared region of spectra shows less background noise compared with the visible region (Debbage and Jaschke 2008; Park et al. 2009).

13.2.2 Magnetic Resonance Imaging

The technique for MRI is based on the principle of precession of the relaxation of hydrogen nuclei in the applied magnetic field and pulses of radiofrequency. When the hydrogen nuclei are exposed to an external magnetic field and pulses of radio frequency are applied, transition of hydrogen nuclei from lower energy to higher energy takes place. After the radio pulses are stopped, the nuclei return to their original state with the emission of radio signals corresponding to resonance of the nuclei undergoing relaxation. There are two types of relaxation processes: T1 (spin–lattice relaxation) and T2 (spin–spin relaxation). The radio signals emitted by these relaxation processes are transformed into images using electronic equipment attached to the MRI system (Grover et al. 2015). In this technique, MRI contrast agents are used, which are discussed in the next section. This technique offers better anatomical resolution than optical imaging and radionuclide imaging (Janib and Moses 2010).

13.2.3 Computed Tomography

Computed tomography involves the use of X-rays, which pass through bone and soft tissues. The difference in the absorption of the X-rays by the tissues produces difference in the contrast, which is converted to images by the instrument. Compared with MRI, CT has poor soft-tissue contrast, which makes it difficult to differentiate

tumours from their surrounding tissue. To overcome this drawback, contrast agents such as iodine, bismuth, barium, and gold are used (Weissleder 2002).

13.3 Ultrasound

Ultrasound is the safest, cheapest and most widely used imaging technique, which utilises high frequency (more than 20,000 kHz) sound waves. A transducer emitting ultrasound is placed on the region of interest covered by gel to ensure good conductance by avoidance of air spaces between the transducer and the site of imaging. The ultrasound emitted by the transducer is reflected by the organs inside the body of the subject and the receiver on the transducer captures the reflected soundwaves and converts them into images using a computer (Massoud and Gambhir 2003).

13.4 Radionuclide-Based Imaging

Nuclear medicine deals with the application of radioisotopes in the diagnosis and/or therapy of different disease conditions. Radio pharmaceutical formulations bearing alpha, beta- or gamma-emitting radioisotopes are administered in vivo and their distribution and pharmacokinetics can be tracked using techniques such as PET and SPECT that record the location and intensity of emission of radioactivity from the radiolabelled compound in different regions of the body and, using sophisticated software algorithms, represent the data as an image of in vivo distribution. This is discussed in the next sections (Janib and Moses 2010).

13.5 Diagnostic/Contrast Agents Used in Medicine

13.5.1 Optical Imaging Contrast Agents

There are various classes of fluorescent dyes such as carbocyanine dyes, tetrapyrrole dyes, fluorescent lanthanide chelates etc. The carbocyanine dyes show visible to near-infrared wavelength absorption and have good fluorescence (450–900 nm). Commercially available dyes of carbocyanine class are the CyDye™ series developed by Amersham Pharmacia Biotech, Germany, which have been used as contrast agents for optical imaging and immune assays. Of this class, indocyanine green is widely used for the screening of tumours, cardiac and hepatic function and vasculature imaging in ophthalmology. The tetrapyrrole class consists of porphyrins, chlorins and phthalocyanines. δ-Aminolevulinic acid, which is a precursor of protoporphyrin IX, an intermediate in the synthesis of haemoglobin, has also been exploited as a contrast agent for the detection of tumours. Fluorescent lanthanide chelates consist of organometallic complexes of lanthanide with macrocyclic

molecules. Other miscellaneous dyes include fluorescein isothiocyanate and Ethyl Nile blue A (Licha 2002).

13.5.2 Contrast Agents for Magnetic Resonance Imaging

The diagnostic agents used in MRI consist of compounds or paramagnetic materials that can alter the relaxation times of protons. The contrast agents used are complexes or compounds of transitional and rare earth metals of a paramagnetic nature, mainly gadolinium-based and non-gadolinium-based. Metal chelates such as gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA), commercially known as Magnevist™, is the most widely used MRI contrast agent. Other gadolinium-based complexes are gadolinium-1,4,7,10-tetracarboxymethyl-1,4,7,10-tetraazacyclododecane (Dotarem™) and Gd-DTPA diamines such as Omniscan™ and Optimark™. Non-gadolinium contrast agents include manganese chloride and its complexes, iron compounds and derivatives and copper derivatives. Metallic nanoparticles of iron, lanthanides or their combination are also employed as contrast agents in MRI for detection of tumours (Gries 2002; Schwert et al. 2002).

13.5.3 Contrast Agents for Computed Tomography

Contrast agents for CT include compounds of elements of high atomic numbers such as bromine, iodine, bismuth and lead. The water soluble contrast agents include strontium bromide and iodine salts of lithium and sodium. Barium sulphate is used as a contrast agent for examination of the gastrointestinal tract, whereas xenon gas is used for assessment of the pulmonary system. Iodine compounds are widely used as contrast agents for X-rays owing to their low toxicity, high hydrophilicity and stability. The inorganic iodide and organic compounds such as mono-iodine compounds (Uroselectan A), bis-iodine (Uroselectan B) and tris-iodine substances (diatrizoate), ionic (diatrizoate), non-ionic (iopromide), monomers (iopromide), dimers (iotrolan) etc. have also been used as contrast agents (Krause and Schneider 2002).

13.5.4 Contrast Agents for Ultrasound

The first generation of contrast agents include microbubbles and macrobubbles, which are air-in-water dispersions. The preparation method of such systems is easy and does not require any complex instrumentation. Plain microbubbles can be prepared using air or inert gas and isotonic saline solution. Some solutes such as dextrose, renografin and indocyanine green are used to lower the surface tension or to enhance the viscosity to improve the stability of microbubbles. The marketed

contrast agent Echovist™ or SHU 454 (Schering), approved for use on the European market, consists of dextran, whereas Levovist™ or SHU 508 (Schering) consist of dextran and palmitic acid, which acts as a surfactant, improving the stability of the microbubble when mixed with the aqueous phase. Albumin has also been used in the development of microbubbles (Albunex™, developed by Molecular Biosystems USA) and approved for use in the USA. It is used for opacification of myocardium. The second generation of contrast agents includes fluorinated gases such as SF_6, C_3F_8, C_4F_{10}, C_5F_{12} or C_6F_{14} with nitrogen in a polymeric impermeable shell (Klibanov 2002).

13.5.5 Radionuclide-Based Imaging/Therapy Agents

A radioisotope is an unstable radionuclide, which undergoe radioactive decay through emission of gamma rays or alpha or beta particulate emissions. Radionuclides with desired properties can be tagged to different molecules for use in clinical application. A radionuclide incorporating formulation of adequate purity and pharmaceutical safety for in vivo administration in humans or animals for the purpose of diagnosis or therapy is known as a radiopharmaceutical (Sood et al. 2004). Table 13.1 enlists some clinically relevant radioisotopes with their primary emissions (Qaim 2001). Typically, a diagnostic imaging technique such as SPECT is performed with gamma-emitters and PET with positron emitters, whereas therapy is the domain of alpha- and beta-emitting radioisotopes (Velikyan 2014).

Table 13.1 Examples of radionuclides used for imaging and therapy (Velikyan 2014; Williams 2016)

Radionuclide	Half life	Emission	E_{max} (keV)	Use
^{18}F	109 min	Positron	250	Imaging (PET)
^{76}Br	16.2 h	Positron	375,427,1530	Imaging (PET)
^{124}I	100.2 h	Positron	686,974	Imaging (PET)
^{68}Ga	68 min	Positron	836	Imaging (PET)
^{67}Ga	3.26 days	Gamma	93,185,300	Imaging (SPECT)
^{99m}Tc	6.01 h	Gamma	140	Imaging (SPECT)
^{111}In	67.3 h	Gamma	171,245	Imaging (SPECT)
^{123}I	13.3 h	Gamma	159	Imaging (SPECT)
^{32}P	14.3 days	Beta	649	Radiotherapy
^{89}Sr	50.5 days	Beta	585	Radiotherapy
^{131}I	8.05 days	Beta Gamma	97,192 364,637	Radiotherapy
^{153}Sm	46.3 h	Beta	200,226,265	Radiotherapy
^{177}Lu	6.73 days	Beta	48,111,149	Radiotherapy
^{188}Re	17.0 h	Beta	728,795	Radiotherapy
^{211}At	7.2 h	Alpha	5870	Radiotherapy
^{213}Bi	46 min	Alpha	5550,5870	Radiotherapy

^{99m}Tc (technetium-99 m) has been widely used for imaging because of its ideal properties such as a convenient half-life of around 6 h and pure monoenergetic gamma emission of 141 keV, which permits high-quality scintigraphic images with minimal radiation dose to the patient. ^{99m}Tc has a versatile chemistry and can be made to achieve several oxidation states (from +7 to +1) by careful selection of ligands and reducing agents. This allows it to be tagged to a wide range of ligands and organic compounds for imaging of almost all the organs in the human body (Shukla et al. 1984). Easy availability of pharmaceutical grade ^{99m}Tc from ^{99}Mo-^{99m}Tc generator with feasibility of quick, simple and user friendly kit based formulations of different ^{99m}Tc radiopharmaceuticals at hospital radiopharmacy has made this artificial radionuclide a "Workhorse" of nuclear medicine.

There are strict regulatory guidelines regarding the quality control of radiopharmaceuticals, their handling storage and dosimetry. The radiopharmaceuticals are assessed for radionuclide purity, chemical purity, physical purity and biological purity, as per the protocols mentioned in the pharmacopoeias and the regulatory authorities applicable to the region (Ziessman et al. 2013).

Especially in case of radiometals, different chelating agents are employed to deliver the radionuclide to the site of interest for the purpose of imaging or therapy. Diethylenetriaminepentaacetic acid (DTPA) is a bifunctional chelating agent that links with the radiometal at one end and the targeting biomolecule at the other end. It is a poor chelator of ^{99m}Tc, but it is mostly used for radioactive lanthanides. 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*′″-tetraacetic acid (DOTA) is also a good chelator for lanthanide series metals. The bifunctional chelator hydrazinonicotinic acid (HYNIC) is also used for radiolabelling of biological targeting ligands with ^{99m}Tc (Liu et al. 2010). The ^{18}F-labelled glucose derivative 2-fluoro-2-deoxyglucose (FDG) accounts for the bulk of PET scans, and is used for imaging of various organs such as the brain and heart and tumours where there is a high utilisation of glucose (Minn et al. 1988).

13.6 Concept of Theranostics in the Treatment of Cancer

The term "theranostics" was devised by Funkhouser in 2002 (Funkhouser 2002). Theranostic refers to the combination of imaging and therapeutic capabilities on a single platform and involves delivery of the imaging or contrast agent and the therapeutic agent in a single dose (Jain et al. 2015). The main objective of theranostics is to monitor the disease along with the treatment, which is essential in cancer to optimise the therapeutic outcome and avoid the adverse effects of conventional drug therapy. To fulfil the objective, various nanocarriers are utilised, which comprise nanoparticles, liposomes, micelles, dendrimers etc. The small size of these nanocarriers is a big advantage as they can easily penetrate into the tumour vasculature and get retained owing to the "enhanced permeation retention" effect (Kelkar and Reineke 2011). The theranostic nanocarriers can be targeted, non-targeted or stimuli-based. The targeted theranostic carriers have a targeting ligand attached to

the carrier that can bind to the overexpressed receptors in tumour cells. Antibodies can also be used to provide cancer-specific targeting, but their immunogenicity and large size can cause poor penetration into tumour cells. Peptides are useful as theranostic carriers as they have a relatively lower size and reduced immunogenicity. The non-targeted theranostic carriers rely mainly on the enhanced permeation effect. The stimuli-based theranostic nanocarriers take the advantage of the tumour microenvironment, such as hypoxia and low extracellular pH (Bhujwalla et al. 2018).

13.6.1 Theranostic Liposomes

Liposomes are spherical vesicles ranging from 30 nm to several micrometres in size and consist of an aqueous core enclosed in lipidic bilayer membranes that are artificially prepared using phospholipids, as shown in Fig. 13.2. Liposomes are biocompatible, biodegradable and are widely used as drug delivery systems as they can encapsulate hydrophilic as well hydrophobic drugs (Akbarzadeh et al. 2013). Liposomes were the first nanocarriers approved by the US FDA for clinical applications (Muthu and Feng 2013). Theranostic liposomes consist of drug and imaging/contrast agent encapsulated within the liposome and have an attached targeting ligand. Yang et al. developed folate-targeted theranostic liposomes of around 105 nm in size and encapsulated fluorescent cadmium telluride (CdTe) quantum dots, which were hydrophilic in nature. The theranostic liposomes showed good anticancer activity when evaluated using the human uterine cervix cancer cell line (HeLa) (Yang et al. 2009). Liposomes containing gadolinium (III) lipid derivative of 92–144 nm in size have also been prepared for MRI as well as photodynamic

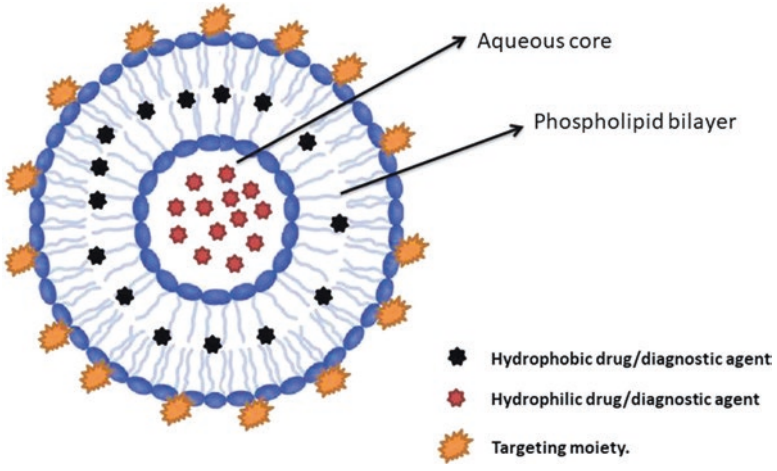

Fig. 13.2 Schematic diagram of a liposome

therapy for the treatment of cancer (Skupin-Mrugalska et al. 2018). Cancer cell membranes were used for liposome preparation and were loaded with tetrakis (4-carboxyphenyl) porphyrin, which is an imaging agent for PET and for use in photodynamic therapy (Yu et al. 2018). Sono-sensitive liposomes of 150 nm in size, loaded with doxorubicin along with gadoteridol, were synthesised for MRI as well as pulsed ultrasound-stimulated drug delivery in tumours (Rizzitelli et al. 2015). Yoon et al. formulated a liposome–microbubble system for ultrasound-triggered gene delivery and the system was conjugated with human epidermal growth factor 2 (HER2) antibody for targeting breast cancer (Yoon et al. 2014).

13.6.2 Theranostic Nanoparticles (Polymeric and Lipid)

Nanoparticles have been widely explored as controlled and targeted drug delivery systems. Nanoparticles can be polymeric, lipidic or inorganic. Polymeric nanoparticles are prepared using biodegradable polymers of natural or synthetic sources. Natural polymers include chitosan, gelatine, albumin etc., whereas synthetic polymers include polylactic acid, poly(lactic-co-glycolic acid) (PLGA), polycaprolactone, polyethyleneimine (PEI) etc (Prasad et al. 2017). The lipid nanoparticles can be classified as solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and lipid drug conjugates (LDCs). Solid lipid nanoparticles are made using solid lipids such as triglycerides and surfactants, but they have low drug-loading capacity. To overcome this drawback, NLCs were developed that consist of solid lipids as well as liquid lipids, which provide high drug loading and low drug expulsion. LDCs are prepared by covalent linkage between functional groups of drug and lipid chains (Wilczewska et al. 2012). Bahmani et al. prepared theranostic polymeric nanoparticles using polyallylamine wherein indocyanine green dye was used as a near-infrared imaging agent and HER2 antibody was linked covalently to target ovarian cancer cells. The size of the prepared theranostic nanoparticles was less than 200 nm and Bahmani et al. were able to induce 86% death in SKOV3 cells in vitro by photothermal destruction by laser irradiation at 800 nm (Bahmani et al. 2014). Jin et al. developed theranostic nanoparticles for targeting triple-negative breast cancer cells that lack oestrogen receptor, progesterone receptor and human epidermal growth factor receptor-2. They synthesised a cyclic arginine-glycine-aspartic acid (cRGD) peptide linked to poly [2-methoxy-5-(2-ethyl-hexyloxy)-1,4-phenylenevinylene], which is a photosensitiser producing reactive oxygen species when exposed to light irradiation and also acts as a contrast agent. The synthesised theranostic nanoparticles demonstrated antitumour activity in nude mice bearing MDA-MB-231 tumours (Jin et al. 2018). Solid lipid theranostic nanoparticles 145 ± 12 nm in size were developed to target $\alpha_v\beta_3$ integrin receptors expressed in tumours using conjugated cyclo-Arg-Gly-Asp-D-Phe-Lys peptide and PdSe-encapsulated quantum dots for near-infrared imaging in vivo and evaluated in nude mice bearing orthotopic MDA-MB-435 tumours (Shuhendler et al. 2012).

Quantum dot-encapsulated SLNs of 130.1 ± 13.4 nm crafted for delivery of siRNA and paclitaxel demonstrated apoptosis in human lung carcinoma cells in vitro along with fluorescence for imaging (Bae et al. 2013).

13.6.3 Theranostic Micelles

Micelles are nanocarriers of 5–100 nm in size prepared by using amphiphilic copolymers or conjugates of soluble copolymers with lipids, as shown in Fig. 13.3. Poorly soluble drugs can be easily incorporated inside the hydrophobic part of micelles and the solubility of such drugs can be enhanced. Polyethylene glycol and polyvinyl alcohol have been used as hydrophilic blocks for the preparation of amphiphilic micelles, whereas for hydrophobic core, L-lysine, aspartic acid, caprolactone, D,L-lactic acid etc are used. In the case of lipidic core micelles, conjugations of phospholipids along with hydrophilic polymers such as PEG are used. Targeted delivery is also possible by attachment of ligands such as transferrin and folate. Cationic lipid-based micelles are used for intracellular trafficking of DNA, whereas micelles loaded with paramagnetic metal complexes are used as diagnostic agents (Torchilin 2007). Kumar et al. prepared theranostic polymer-lipid micelles of less than 50 nm in size for delivery of doxorubicin along with loading of photoluminescent quantum dots of cadmium selenide (CdSe) for imaging (Kumar et al. 2012). Yang et al. prepared theranostic polymeric micelles of less than 200 nm in size using copolymers of monomethoxy poly(ethylene glycol), alkylamine-grafted poly(L-aspartic acid) and carbocyanine dyes for near-infrared imaging and photodynamic therapy of cancer in A549 tumour-bearing nude mice (Yang et al. 2013).

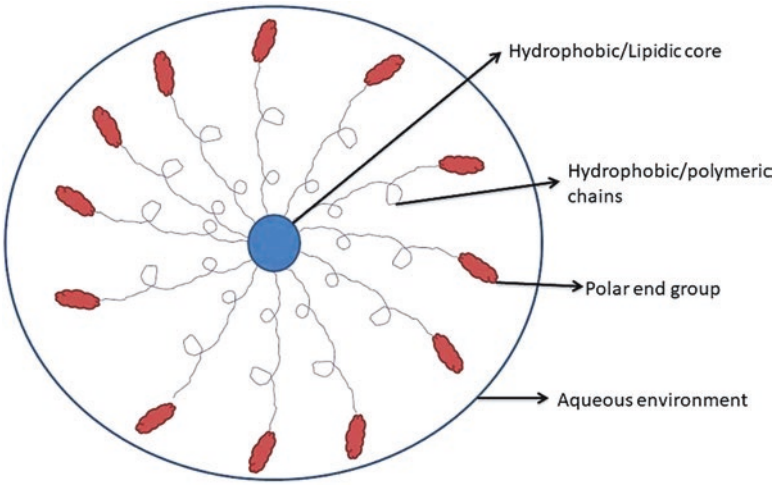

Fig. 13.3 Schematic diagram of a micelle

Upponi and co-workers developed polymeric micelles of 16 nm size using PEG 2000–phospholipid complex. They encapsulated paclitaxel as a therapeutic agent and superparamagnetic iron oxide nanoparticles as an imaging agent for MRI and evaluated in tumour-bearing mice (Upponi et al. 2018). Micelles have also been engineered for gene delivery. Chen et al. synthesised pH-sensitive micelles using ternary block polymer and PEG for delivery of doxorubicin and siRNA, which enhanced apoptosis in human ovarian cancer SKOV-3 cells (Chen et al. 2014).

13.6.4 Theranostic Dendrimers

Dendrimers were first reported by Vögtle et al. as "cascade molecules", and later Tomalia and Fréchet named them dendrimers (Tomalia and Fréchet 2002). Dendrimers are synthetic macromolecules, nanometric in size and have a well-defined structure, consisting of three different parts or domains: (1) the central core consisting of a group of atoms with at least two functionalities; (2) the branches that arise from the central core; and (3) terminal groups on the outer surface attached to the branches, as shown in Fig. 13.4. The synthesis of the dendrimers is done stepwise. There are generally two approaches for synthesis, divergent and convergent. In the divergent approach, the synthesis starts from the multifunctional core followed by the addition of monomers having one reactive group and two inactive groups, whereas in the convergent approach, synthesis takes place by joining outer branches with joining units that have two or more active sites and one inactive site (Nimesh 2013). The first dendrimers were synthesised using polyamidoamines. PEI

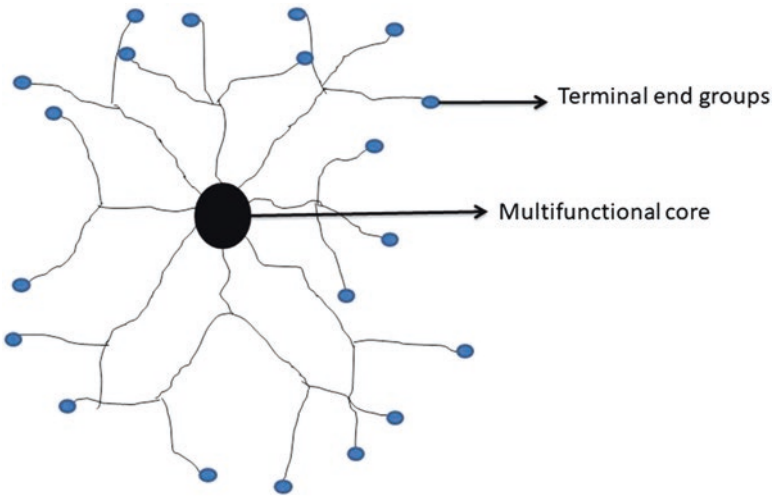

Fig. 13.4 Schematic diagram of a dendrimer

dendrimers were also developed later and made commercially available (Klajnert and Bryszewska 2001). Dendrimers are used as carriers for anti-cancer drug delivery, gene delivery and diagnostic imaging (Abbasi et al. 2014). Majoros et al. prepared multifunctional theranostic dendrimers using PAMAM conjugated with fluorescein isothiocyanate imaging agent and folic acid as a targeting moiety (Majoros et al. 2005). Filippi and co-workers synthesised theranostic dendrimer using phospholipid conjugated polyethylene glycol (PEG) and ethylene glycol (EG), GdDOTAGA $(C18)_2$ as the MRI agent and prednisolone phosphate as the drug. The prepared dendrimers were evaluated in murine melanoma tumour models (Filippi et al. 2017). The dendrimers prepared using PAMAM and ligands such as RGD, folic acid and thioaptamer, have been conjugated for delivery of siRNA, miRNA and DNA for targeted delivery to cancer cells (Abedi-Gaballu et al. 2018).

13.6.5 Theranostic Inorganic Nanoparticles

This class of nanoparticles consists of calcium phosphate-based nanoparticles, metallic nanoparticles, quantum dots, carbon nanotubes and silica-based nanoparticles, as shown in Fig. 13.5. Inorganic nanoparticles are more stable than organic

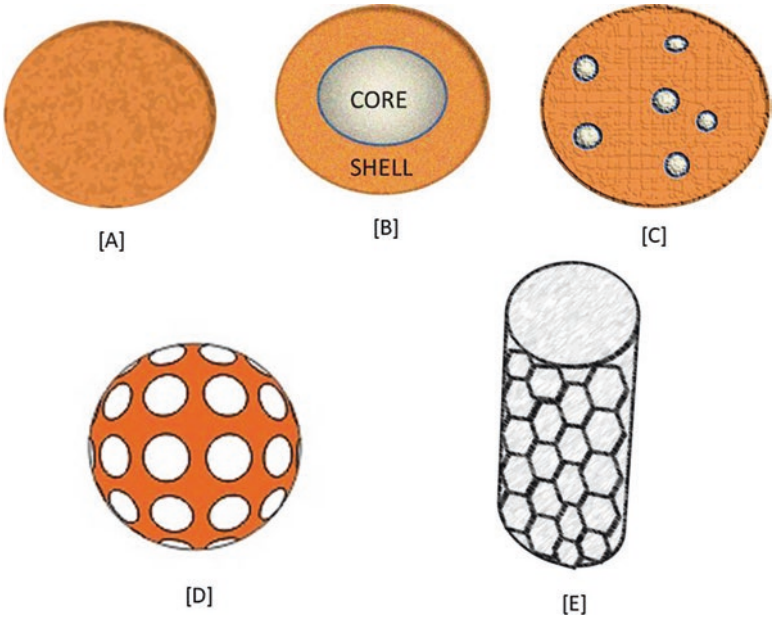

Fig. 13.5 Inorganic nanoparticles. (**A**) Metallic nanoparticles, (**B**) core shell metallic nanoparticles, (**C**) lanthanide-doped nanoparticles, (**D**) mesoporous silica nanoparticles, (**E**) carbon nanotubes

ones and are used for imaging and drug delivery. Each class of inorganic nanoparticles has its own advantages and disadvantages. Calcium phosphate-based nanoparticles are biocompatible, non-toxic and non-immunogenic and resemble human mineralised tissue. They have pH-dependent solubility and remain intact at physiological pH values, but solubilise at low pH (less than 5) found in endosomes and lysosomes after cell uptake. Calcium-based nanoparticles consist of nanocrystalline hydroxyapatite or amorphous calcium phosphate. Calcium phosphate nanoparticles can be modified by coating them with a layer of organic compounds or polymers and can be doped with lanthanides for imaging capabilities, which can also be done by encapsulating fluorescent dyes (Degli Esposti et al. 2018). Calcium phosphate nanoparticles with ferucarbotran containing immobilised heparin and ATP as a theranostic platform were synthesised for drug delivery as well as MRI (Nakamura et al. 2018). Haedicke et al. prepared multifunctional calcium phosphate nanoparticles of around 200 nm in size consisting of temoporfin as a photosensitizer, RGDfk peptide for tumour-targeting and DY682-NHS for near-infrared fluorescence imaging.

A decrease in tumour volume and vascularisation was noted when the nanoparticles were administered in tumour-bearing CAL-27 mice owing to photodynamic therapy and apoptosis (Haedicke et al. 2015). Metal nanoparticles consist of a variety of groups such as iron oxide-based nanoparticles, noble metal nanoparticles, nanoalloys and core shell nanoparticles. Iron nanoparticles are the most exploited theranostic nanocarriers and consist of ferromagnetic magnetite (Fe3O4) and ferrimagnetic maghemite (γ-Fe2O3) and hematite (α-Fe2O3). Iron nanoparticles smaller than 20 nm exhibit superparamagnetism. The superparamagnetic iron oxide nanoparticles (SPIONs) are biocompatible and are used as drug delivery platforms and T2 MRI imaging agents. They can also induce hyperthermia in the presence of a magnetic field, which is useful in the destruction of tumour cells along with the production of reactive oxygen species (ROS) and are also useful in photodynamic therapy. Dextran-coated iron oxide nanoparticles (Feridex™) have been approved by the US FDA as a diagnostic agent. Lanthanide oxide nanoparticles and manganese oxide nanoparticles are also used as MRI contrast agents (Xu et al. 2012). Iron oxide nanoparticles with lanthanide doping have been synthesised for T1 and T2 dual MRI (Xu et al. 2013; Douglas et al. 2016; Petran et al. 2018). Iron oxide-based or noble metal nanoparticles are coated with polymers such as polyacrylic acid, PEI, PEG, chitosan etc., which can be modified with a variety of targeting ligands for imaging as well as delivery of anticancer drugs, miRNA, siRNA or DNA (Steitz et al. 2007; Kievit et al. 2009; Santra et al. 2009; Heo et al. 2012; Taylor and Sillerud 2012; Yin et al. 2014).

Quantum dots and carbon nanotubes are emerging platforms for theranostics. Quantum dots are semiconductor nanocrystals 2–10 nm in size, which are composed of elements of groups III to VI. CdSe and CdTe are examples of quantum dots. The quantum dots have the property of exhibiting fluorescence in ultraviolet to near-infrared regions. Quantum dots can be encapsulated in other nanocarriers such

as liposomes and nanoparticles and variety of ligands such as cancer-specific immunoglobulins, specific cancer markers, peptides and monoclonal antibodies can be conjugated for theranostic applications. Carbon nanotubes are allotropes of carbons and are a subset of the fullerene family. They are of two types, single-walled and multi-walled and can be conjugated with anticancer drugs (such as paclitaxel, carboplatin, doxorubicin, oxaliplatin, cisplatin, etoposide etc.), targeting ligands (such as monoclonal antibodies and folate derivatives) and fluorescent probes and radiometal chelates can also be attached for theranostic applications (Ali-Boucetta et al. 2008; Cai et al. 2005; Chaudhuri et al. 2009; Chen et al. 2008; Degim et al. 2010; Dhar et al. 2008; Hampel et al. 2008; Lay et al. 2010; Levi-Polyachenko et al. 2009; Li et al. 2010; Liu et al. 2008; Liu et al. 2009; Mahmood et al. 2009; McDevitt et al. 2007; Ou et al. 2009; Podesta et al. 2009; Tan et al. 2011).

Silica nanoparticles consist of solid silica nanoparticles (SiNPS) as well as mesoporous silica nanoparticles (MSNs). Modified solid silica nanoparticles ranging in size from 20 to 100 nm are used for imaging as well as drug delivery. Mesoporous silica nanoparticles are different from solid silica nanoparticles in that they have large surface areas, varying pore sizes and pore volumes. They are used to entrap imaging agents as well as a variety of drugs and range in size from 60 to 1000 nm. The grafting of functional groups is possible on the surface. Core shell-type nanoparticles have also been prepared with metallic nanoparticles inside mesoporous silica shell (Vivero-Escoto et al. 2012).

Goel et al. prepared mesoporous silica nanoparticles measuring 80 nm for targeted delivery of the anti-vascular endothelial growth factor (VEGF) agent sunitinib by modification of MSNs with polyethylene glycol and radioisotope ^{64}Cu for in vivo imaging. It was found that a higher amount of sunitinib was delivered to U87MG human glioblastoma tumour-bearing mice compared with non-targeted counterparts (Goel et al. 2014). Cheng et al. developed trifunctional mesoporous silica nanoparticles incorporating a near-infrared fluorescent contrast agent, ATTO647N and photosensitiser (Pd-porphyrin) into the pores, whereas cRGDyK peptides were conjugated outside to target overexpressed $\alpha_v\beta_3$ receptors in cancer cells (Cheng et al. 2010).

13.7 Toxicological Aspects

Before the development of theranostic nanoparticles, the contrast agents were used frequently independently in various diagnostic techniques. However, many of them are associated with toxicity issues. Fluorescent dyes such as fluorescein were found to inhibit neuronal growth at a concentration of 0.2 mg/ml when injected into excised chicken retina. They were also found to irritate nerve roots when injected intrathecally and were toxic to patients in high doses. Indocyanine green has been known to show cytotoxic effects in vitro by intracellular accumulation and inhibition of mitochondria (Alford et al. 2009). The contrast agents used in ultrasound were found to be relatively non-toxic compared with those used in other techniques

(Paefgen et al. 2015). Iodine-based contrast agents used for CT and gadolinium-based contrast agents used for MRI are known to cause nephropathy and kidney failure and should be used with caution in patients with renal impairment (Hasebroock and Serkova 2009). Radiopharmaceuticals should be used with extreme care in pregnant women and paediatric patients with proper dosimetry (Ziessman et al. 2013).

The development of theranostic nanoparticles has opened up new dimensions in the treatment of cancer and personalised medicine, but only a few nanoformulations have been approved by the FDA owing to concerns regarding toxicity, which may or may not arise with their long-term use. The nanoformulations need to undergo stringent characterisation to evaluate their toxicity and establish their safety and biocompatibility.

Polymeric nanoparticles prepared using biodegradable materials such as PLGA demonstrated minimal toxicity compared with their counterparts non-biodegradable polymers such as polyacrylates, which showed considerable toxicity owing to the chemical properties of the polymer (Lopalco and Denora 2018). It was observed that PEGylation of the nanoparticles showed a decrease in the toxicity. Cationic lipids and polymers used in the nanopreparations for gene delivery have interactions with critical enzymes such as protein kinase C. To overcome this problem, polymers that degrade at a low pH have been developed. Low-molecular-weight PEI, as well as PEGylation, has shown lower toxicity than high-molecular-weight PEI.

The use of metallic nanoparticles has been a controversial topic. Divalent metal ions in small concentrations can cause nephrotoxicity as they accumulate in the kidneys. Cadmium and selenium used in quantum dots can cause toxicities in vertebrates. Under oxidative stress, cadmium ions bind to sulphydral groups in mitochondria and disrupt its function. Cationic gold nanospheres can cause disruption of anionic cell membranes and lead to toxicity. Silver nanoparticles have shown oxidative stress in the brain and liver by reducing the respiratory chains of mitochondria. Iron oxide nanoparticles can promote oxidative stress and accumulate in target tissues leading to toxicity. Carbon nanotubes can be classified as single-walled carbon nanotubes, as well as multiwall carbon nanotubes. Single-walled carbon nanotubes are relatively non-toxic owing to their small size, whereas multi-walled carbon nanotubes show aggregation in tissues, leading to toxicity. Uncoated grapheme oxide causes in vivo blood clots, pulmonary oedema and granuloma after accumulation in the lungs upon intravenous administration. Mesoporous silica nanoparticles, despite reduced toxicity, can still induce cytotoxicity associated with oxidative stress and cause cardiovascular disorders such as bradycardia and cardiac toxicity. Silica nanoparticles can also inhibit gene expression by the formation of protein agglomerates and can interfere in cell growth and replication. Thus, it is more important to design theranostic nanocarrier systems in such a way that their toxicity can be minimised while the therapeutic and diagnostic potential can be enhanced for better treatment of cancer. This involves rational selection of nanocarrier, diagnostic and therapeutic agents, method of preparation, particle size optimisation, modification of surface properties etc. (Kang et al. 2015).

13.8 Conclusion

The development of nanocarriers is a breakthrough in the field of medicine that has changed the concepts of drug delivery and treatment of various diseases. Cancer, being a global epidemic and a leading cause of death worldwide, requires a platform capable of drug delivery and diagnostic capabilities, which has triggered the development of targeted theranostic nanosystems. Theranostic nanosystems are like a "double-edged sword" because of dual imaging and diagnostic capabilities on a single platform. A wide range of different imaging agents such as dyes, radiolabelled compounds, MRI agents can be accommodated on a nanocarrier along with anti-cancer agents such as drugs, miRNA, siRNA and DNA for therapy. Theranostic nanosystems obtain their targeting ability by the conjugation of various ligands and peptides that can bind to the overexpressed receptor on the cancerous cells. This combination gives a better treatment option along with imaging to monitor the progression of treatment, which is essential for making critical decisions affecting the safety of the patient and the efficacy of the treatment. Toxicity is the main concern in the development of smart targeted theranostic nanosystems, but can be overcome by proper designing of the nanocarrier and its functionalisation. Many developed theranostic formulations are under development and may become available commercially in the near future if they are proven to treat cancer in a safe and effective way.

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Chapter 14
Biosynthesis, Mechanisms, and Biomedical Applications of Silver Nanoparticles

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Abstract Nanoparticles (NPs) can be developed to improve drug penetration and reorient chemotherapy, or selectively target the cancer cells or cell compartment. Both passive and active targeting strategies are used to redirect the anticancer drugs. Noble metals such as the silver NPs (AgNPs) are characterized by electrical, optical, and thermal properties, and can be integrated into products for optical, biological and chemical sensor applications such as pastes, conductive inks, and fillers for high stabilization, electrical conductivity, and low sintering temperatures. The biosynthesis of AgNPs, making use of bacteria, fungi, actinomycetes, yeast, algae, and plants, is eco-friendly, green, nontoxic and inexpensive. The AgNPs sytnhesized are of various shapes and sizes. The AgNPs have diverse bioactivities including antibacterial, antifungal, antiviral, anti-inflammatory, anti-angiogenic, and anticancer activities, with great potential for use in cancer diagnosis and therapy. The mechanisms of AgNP-induced cytotoxicity include endoplasmic reticulum stress, lactate dehydrogenase leakage, and enhanced reactive oxygen species level. Co-application of AgNPs and natural products could play an essential role in nanoscience and nanotechnology, especially in nanomedicine for cancer diagnosis and therapeutics.

Keywords Anti-cancer · Nanobiotechnology · Nanocarrier · Nanomedicine · Silver Nanoparticles

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_14

14.1 Introduction

Nanoparticles (NPs) and nanomaterials can be utilized for human medical applications, including for the delivery of therapeutic drugs to cells, or for the imaging of tissues and organs. NPs are divided into organic and inorganic materials. Organic NPs include the carbon NPs (fullerenes) and inorganic NPs include the magnetic, noble metals (such as gold and silver) and semi-conductive (such as titanium dioxide and zinc oxide) NPs. The inorganic NPs have superior material features with functional versatility and have potential for application in imaging tools as well as for disease treatment owing to their sizes and their benefits as pharmaceutical agents and chemotherapy drugs. Mesoporous silica is established along with the molecular machinery to be used for imaging and as release systems. NPs have been used successfully for the delivery of therapeutic agents (Zhang et al. 2008), in diagnostics for chronic diseases (Hong et al. 2008), and the treatment of bacterial skin infections and burn wounds (Rai et al. 2009). Gold NPs are widely used in imaging, as drug carriers, and in the thermal treatment of biological targets (Cheon and Underwood 2009). The bactericidal behavior of NPs is attributed to the presence of electronic effects as a result of changes in the local electronic structure of the surface owing to their smaller sizes. The NPs become attached to the cell membrane and penetrate inside the bacteria.

Several NPs that are loaded with drugs interact with the organs and tissues and are eventually taken up by the cells. The tissue, cell and cell organelle distribution of the drugs can therefore be controlled and improved by entrapping them into the colloidal NPs, such as the nanocontainer or nanocarrier (Minchinton and Tannock 2006; De Jong and Borm 2008; Zhang et al. 2013). However, not all nanocarriers penetrate tumor tissue (Lammers et al. 2012). Nanomedicine compounds can be designed to improve drug penetration and reorient the chemotherapy or target the cancer cells or cell compartment with the compounds selectively. Both negative and effective targeting strategies are used to redirect the anticancer drugs (Wicki et al. 2015). The nanomedicine treatments can increase the circulatory time of the compound and mediate the release of stimulant-responsive drugs as well as the absorption of the stimulant medication. This may result in reduced tumor cell resistance against targeted NPs (Huwyler et al. 2002; Hu and Zhang 2009). NP drug delivery systems have great advantages such as delivery through the smallest capillary vessels owing to their small sizes and avoiding fast clearance by phagocytes, infiltration of the cells and tissue gap to reach the target site. Existing controlled release features such as the pH, ion, and/or temperature sensitivity of the substance can improve the efficacy of the drugs, whilst minimizing the toxic side effects (Zhang et al. 2010a, b).

Nanobiotechnology combines the nanotechnology area with microbiology, chemistry and physical sciences, and the synthesis of NPs by utilizing the biological systems such as plants, bacteria, and fungi (Ahmad et al. 2003; Prasad 2014; Prasad

et al. 2016, 2018). NPs exhibit new or improved properties based on the specific characteristics such as size, distribution, and morphology, resulting in rapid and tunable applications of the NPs and nanomaterials (Dakhil 2017). The common methods for the synthesis of NPs include physical and chemical approaches using laser ablation, pyrolysis, lithography, chemical vapor deposition, sol-gel techniques, and electro-deposition, but these are expensive and hazardous (Vijayakumar et al. 2013). Different reactants are used, especially reducing agents such as sodium borohydride (Kim et al. 2007), potassium bitartrate (Tan et al. 2003), methoxypolyethylene glycol (Zewde et al. 2016), and hydrazine (Li et al. 1999). It also requires a stabilizing agent such as sodium dodecyl benzyl sulfate or polyvinyl pyrrolidone to prevent the agglomeration of metallic NPs. Generally, the chemical methods are low-cost for high volume, but may involve contamination from the precursor chemicals, the use of toxic solvents, and the generation of hazardous by-products (Thakkar et al. 2010).

There is an increasing need to develop simple, cost-effective, high-yield, and eco-friendly procedures (Gurunathan et al. 2013a, b). The alternative green method for the biosynthesis of metal NPs is via the living organisms or material of biological origin. NPs can be synthesized by using living bacteria or fungi, or plant extracts, which is environmentally friendly, takes place around room temperature or lower, and requires little intervention or input of energy (Dash 2013). The important three factors are: (a) the solvent, (b) the reducing agent, and (c) the nontoxic material. The availability of amino acids, proteins, or secondary metabolites could facilitate the synthesis process, prevent particle aggregation, and is pollution-free. The biological methods using bacterial protein or plant extracts as reducing agents allow control of the particle size, shape, and monodispersity of the NPs, which are important for various biomedical applications (Gurunathan et al. 2009, 2014). The availability of a vast array of biological resources, a decreased time requirement, high density, stability, and the ready solubility of the prepared NPs in water (Thakkar et al. 2010), confer major advantages over the chemical synthetic route of other metallic-based anticancer agents (Caroling et al. 2013; Chaudhari et al. 2012; Yazdi et al. 2015; Jaffat et al. 2017). Many microbes, both unicellular and multicellular, produce inorganic materials, either intra- or extracellularly. Bacteria, yeast, and fungi play important roles in the remediation of toxic metals through the reduction of metal ions and act as nanofactories (Prasad et al. 2016). These microbes are extremely good candidates in the synthesis of cadmium, gold, and silver nanoparticles (AgNPs; Table 14.1) (Jeevan et al. 2012). Extracellular synthesis of NPs occurs outside the bacterial cell. These NPs, spherical, disk, cuboidal, hexagonal, or triangular shaped, have been synthesized using cells, culture supernatant, or aqueous cell-free extract (Klaus et al. 1999; Srivastava and Constanti 2012; Oves et al. 2013; Singh et al. 2013). The NPs are collected as pellets, which can be dissolved in suitable solvent. The extracellular methods are more useful than the intracellular methods because of the ease of obtaining the NPs from the solution (Singh and Shedbalkar 2015).

Table 14.1 Biosynthesis of silver nanoparticles (AgNPs) from different bacterial species

Bacteria species	Shape	Size (nm)	Biosynthesis	Activities	References
Gram-negative					
Acinetobacter calcoaceticus	Spherical	8–12	Extracellular	Enhanced antibacterial activity	Singh et al. (2013)
		4–40	Extracellular	Antibacterial	Gaidhani et al. (2013)
Aeromonas sp. SHlO	–	6.4	Extracellular and intracellular	–	Mouxhg et al. (2006)
Bordetella sp.	–	63–90	Extracellular	Antibacterial	Thomas et al. (2012)
Enterobacter aerogenes	Spherical	25–35	Extracellular	–	Karthik and Radha (2012)
Escherichia coli	Spherical	42.2–89.6	Extracellular	–	Gurunathan et al. (2009)
Escherichia fergusonii	Spherical	10–50	Extracellular	Cytotoxicity against MCF-7 cells	Gurunathan et al. (2013a, 2013b)
Geobacter sulfurreducens	–	–	Extracellular	–	Law et al. (2008)
Gluconobacter roseus	–	10	Extracellular	Cytotoxic effects on platelets	Krishnaraj and Berchmans (2013)
Idiomarina sp. PR58-8	–	25	Intracellular	–	Seshadri et al. (2012)
Klebsiella pneumoniae	Spherical	15–37	Extracellular	Antibacterial	Kalpana and Lee (2013)
	–	5–32	Extracellular	Enhanced antibacterial activity	Shahverdi et al. (2007)
Morganella spp.	Quasi-spherical	10–40	Extracellular	–	Parikh et al. (2011)
Morganella psychrotolerans	Spherical	70–100	Extracellular	–	Ramanathan et al. (2011)
Proteus mirabilis	Spherical	10–20	Extracellular and intracellular	–	Samadi et al. (2009)
Pseudomonas aeruginosa	Spherical, disk-shaped	6.3 ± 4.9	Extracellular	–	Kumar and Mamidyala (2011)
	Quasi-spherical	5–25	Intracellular	–	
Pseudomonas stutzeri AG259	Triangular, hexagonal, and spheroidal	200	Cell poles	–	Klaus et al. (1999)

Rhodobacter sphaeroides	Spherical	3–15	Extracellular	–	Bai et al. (2011)
Shewanella oneidensis MR-1	Spherical	2–16	Extracellular	–	Debabov et al. (2013)
Stenotrophomonas maltophilia	Cuboidal	93	Extracellular	Antimicrobial and anti-cancer	Oves et al. (2013)
Vibrio alginolyticus	Spherical	50–100	Extracellular and intracellular	–	Rajeshkumar et al. (2013)
Xanthomonas oryzae pv. *oryzae* BXO8	Spherical, triangular, rod-shaped	14.86	Extracellular	–	Narayanan and Sakthivel (2013)
Gram positive					
Bacillus sp.	–	5–15	Extracellular and periplasmic	–	Pugazhenthiran et al. (2009)
Bacillus subtilis	Triangular, hexagonal	–	Extracellular	–	Kannan et al. (2011)
Bacillus subtilis	Spherical	20–60	Intracellular	Antifungal activity	Paulkumar et al. (2013)
Bacillus thuringiensis	–	43.52–142.97	Extracellular	Larvicidal activity against *Aedes aegypti*	Banu et al. (2014)
	Spherical	–	intracellular	Antimicrobial	Dash (2013)
Brevibacterium casei	Spherical	10–50	Intracellular	Anti-coagulant effect	Kalishwaralal et al. (2010)
Corynebacterium SH09	–	10–15	Extracellular	–	Zhang et al. (2005)
Staphylococcus aureus	–	160–180	Extracellular	Antibacterial	Nanda and Saravanan (2009)
Exiguobacterium sp. KNU1	Spherical	5–50	Extracellular	Antibacterial	Tamboli and Lee (2013)
Geobacillus stearothermophilus	Spherical	5–35	Extracellular	–	Fayaz et al. (2011)
Lactobacillus mindensis	Spherical	2–20	Extracellular	–	Dhoondia and Chakraborty (2012)
Lactobacillus mixture	Spherical	30–100	Extracellular	Antioxidant	Dakhil (2017)
Lactobacillus acidophilus 58p	Spherical	30.65 ± 5.81	Extracellular	Antimicrobial	Garmasheva et al. (2016)
Lactobacillus plantarum 92 T	Spherical	19.92 ± 3.4	Extracellular	Antimicrobial	
Pediococcus pentosaceus	–	–	Intracellular	–	Sintubin et al. (2009)
Rhodococcus NCIM 2891	Spherical	30	Extracellular	–	Otari et al. (2014)

14.2 Silver Nanoparticles

Silver nanoparticles (AgNPs) are important because of their unique properties (Klaus-Joerger et al. 2001). AgNPs have gained special interest over gold and copper NPs because of their surface plasmon resonance (SPR) energy, which is located away from the interband transition energy (El-sheekh and El-kassas 2016). Because of their catalytic, optical, electrical, and magnetic properties, AgNPs have applications in electronic components, biosensors, environmental remediation, antimicrobial and anticancer agents, cosmetic products, optical catalysis, drug delivery (Klaus-Joerger et al. 2001; Kasthuri et al. 2009; Dubey et al. 2010; Nabikhan et al. 2010; Nithya and Ragunathan 2012; Aziz et al. 2014, 2015, 2016, 2019; Hussein et al. 2020), spectrally selected coatings for solar energy absorption, intercalation material for electrical batteries, optical receptors, chemical catalysis, and bio-labeling (Kalimuthu et al. 2008). AgNPs have been used extensively in household utensils, health care industry, and in food storage, environmental, and biomedical applications such as antibacterial, antifungal, antiviral, anti-inflammatory, anticancer, and anti-angiogenic products (Fig. 14.1) (Zhang et al. 2016). The AgNPs-based products have been approved by a range of accredited bodies, including the US FDA, US EPA, SIAA of Japan, Korea's Testing and Research Institute for the Chemical Industry, and the FITI Testing and Research Institute (Abou El-Nour et al. 2010).

14.2.1 Biosynthesis

Silver nanoparticles have been biosynthesized using methods including the chemical reduction of silver (Ag) ion in aqueous solution (Liz-Marzán and Lado-Touriño 1996), photo-reduction (Pileni 2000; Sun et al. 2001), thermal decomposition in organic solutions (Esumi et al. 1990), and laser radiation (Henglein 1993, 1998). However, these are expensive, unstable, and also the AgNPs are toxic and cause

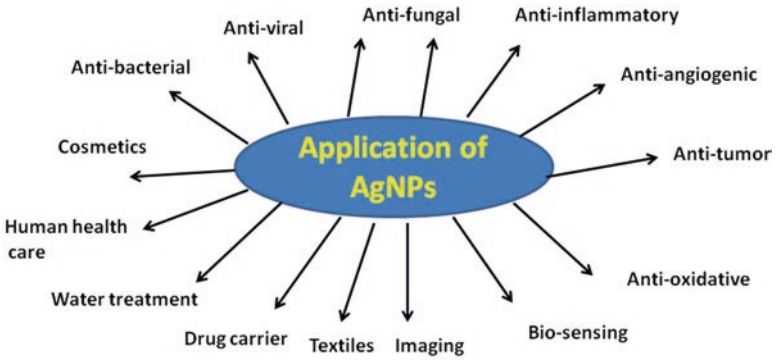

Fig. 14.1 Applications of silver nanoparticles (AgNPs) (Adapted from Zhang et al. 2016)

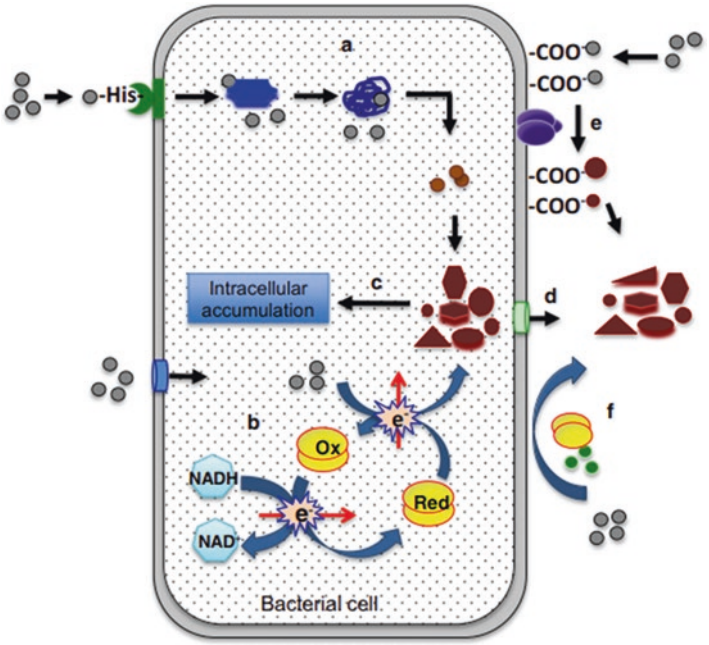

Fig. 14.2 Proposed mechanism of AgNP synthesis (Adapted from Singh and Shedbalkar 2015). (**a**) Cellular uptake of silver ions and activation of silver reduction machinery, (**b**) electron shuttle system involving various cofactors and enzymes, (**c**, **d**) intra-or extracellular localization of AgNPs, (**e**) electrostatic interaction between silver ions and cell wall components, and (**f**) reduction through extracellular enzymes and other organic molecules released into the solution

several side effects and may not be suitable for medical or pharmaceutical purposes (Omidi et al. 2014). The development of biomedical applications has led to the need for a more reliable, nontoxic, and eco-friendly methods of NP synthesis (Braydich-Stolle et al. 2005). Although the log time of used AgNPs has been established, the evidence for silver toxicity is still not clear (Abou El-Nour et al. 2010). Figure 14.2 shows the proposed mechanism of bacteria-mediated synthesis of AgNPs: (a) cellular uptake of silver ions and activation of silver reduction machinery, (b) electron shuttle system involving various cofactors and enzymes, (c, d) intra- or extracellular localization of AgNPs, (e) electrostatic interaction between silver ions and the cell wall components, and (f) reduction through extracellular enzymes and other organic molecules released in the solution (Singh and Shedbalkar 2015). The bacteria may use nitrate anion (NO^{3-}) as a source of nitrogen, leaving behind the metallic Ag ion (Dash 2013). The extracellular synthesis of AgNPs using *Lactobacillus* species is low-cost and effective (Chaudhari et al. 2012). For intracellular synthesis, the bacterial cells are added to the culture medium containing the silver salt and incubated at proper conditions of growth, and the cells are resuspended in sterile distilled water before challenging with silver salt to avoid contamination by the media components

(Singh and Shedbalkar 2015). To obtain AgNPs using intracellular methods, the cells are ultrasonicated (Kalishwaralal et al. 2010). Heat treatment such as autoclaving and the detergents and salts can also be employed to lyse the cells (Fesharaki et al. 2010; Krishnamurthy and Yun 2013). Hence, it is more complicated than the extracellular method.

14.2.2 Characterization

The characterization of NPs is important in understanding and controlling the NP synthesis and applications. Different techniques such as transmission and scanning electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy, powder X-ray diffractometry (XRD), Fourier transform infrared spectroscopy, and ultraviolet–visible (UV–Vis) spectroscopy are used to characterize the NPs (Abou El-Nour et al. 2010). The parameters to be determined are the particle size, shape, crystallinity, fractal dimensions, pore size, surface area, orientation, intercalation, and the dispersion of NPs and nanotubes in the nanocomposite materials (Zewde et al. 2016). The morphology and particle size can be measured using TEM, SEM, and AFM. The DLS determines the particle size distribution. XRD is used for the determination of crystallinity, and UV–Vis spectroscopy is used to confirm the sample formation based on the plasmon resonance (Abou El-Nour et al. 2010).

14.2.3 Anti-microbial Activities

The antibiotic-resistant microbes have become a major global concern. It is important to develop newly effective antimicrobial agents that can overcome the multiple antibiotics resistance of the microorganisms (Franci et al. 2015). AgNPs are considered to be novel agents for antimicrobes (Vijayakumar et al. 2013), with good antimicrobial and antioxidant activities (Niraimathi et al. 2013), antifungal, anti-inflammatory, antiviral, anti-angiogenesis, and antiplatelet activities (Caroling et al. 2013). Low concentrations of AgNPs may have no cytotoxicity on human cells, but may be deadly for many viruses and bacteria. The AgNPs may possibly reduce the toxicity on the cells, without affecting the antibacterial efficacy (Karimzadeh and Mansour 2010).

The high antibacterial activity of AgNPs compared with other salts is attributable to their finely sharp surface and extremely large surface area. The antibacterial action of AgNPs (Fig. 14.3) has been proposed as follows:

(a) The small AgNPs penetrate through the cell membrane and create pores to cause cellular leakage.
(b) The intracellular processes are disturbed to provide better contact with microorganisms. The bacterial membrane contains sulfur-containing proteins and the AgNPs interact with these proteins in the cell as well as with the phosphorus-

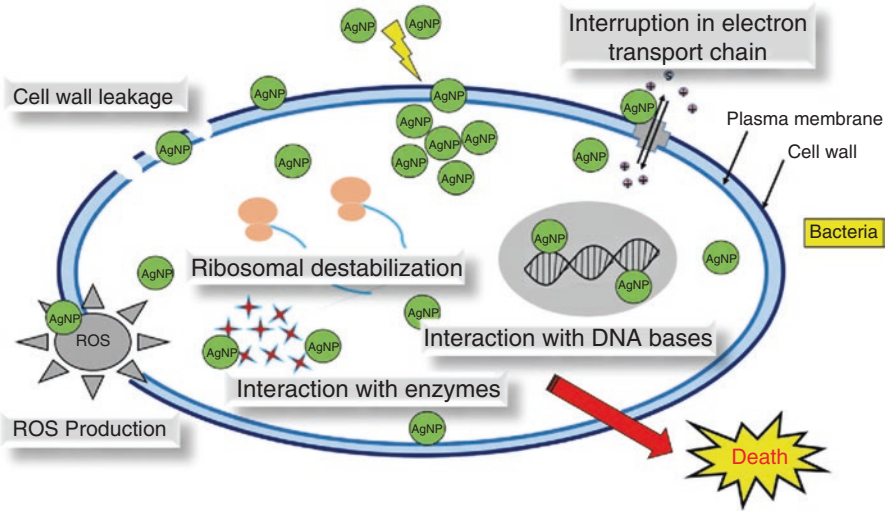

Fig. 14.3 Antimicrobial action of silver nanoparticles (AgNPs) (Adapted from Patil and Kim 2017)

containing compounds such as DNA. Inside the bacterial cell, the AgNPs form a low-molecular-weight region in the center of the bacteria to which the bacteria conglomerates, thus protecting the DNA from the Ag ions.

(c) The AgNPs break the dsDNA.
(d) DNA replication is inhibited.
(e) Interaction with 30S ribosome.
(f) Inactivation of vital enzymes.
(g) Protein is denatured.
(h) Cellular signaling is modulated.
(i) Reactive oxygen species (ROS) is generated, which acts on the DNA and cell membrane.
(j) Ag ions are released, which affect the normal functioning of membrane proteins, and enhance their bactericidal activity. The AgNPs destabilize the plasma membrane potential and deplete the levels of intracellular adenosine triphosphate (ATP) by targeting the bacterial membrane.
(k) Accumulation inside the cells in lethal concentrations results in bacterial cell death (Patil and Kim 2017).

The mechanisms of AgNP-induced cell death are observed in *E. coli* through the leakage of reducing sugars and proteins. The AgNPs destroy the permeability of the bacterial membranes via the generation of many pits and gaps, indicating the damage to the bacterial cell membrane structure (Dibrov et al. 2002; Li et al. 2010; Patil et al. 2012). The AgNPs have greater affinity for the interaction with phosphorous and sulfur-containing biomolecules found in the extracellular (membrane protein), and the intracellular components (DNA bases, protein), which are involved in cell division, respiration, and cell survival (Patil and Kim 2017). The Ag ions display antibacterial activity by interacting with the peptidoglycan cell wall and plasma

membrane (Radzig et al. 2013) and also by inhibiting bacterial DNA replication through the reaction with sulfhydryl groups in the protein (Seth et al. 2011). The Ag ion can damage the protein structures of the bacteria by binding to the thiol and amino groups (Choi et al. 2008). The interaction of the NPs with the thiol group leads to the stimulation of ROS, resulting in the inhibition of respiratory enzymes and then cell death (Holt and Bard 2005; Ninganagouda et al. 2014).

The AgNPs biosynthesized by using *Abutilon indicum* leaf extract exhibit greater antibacterial effects (inhibition zone diameter) on *Staphylococcus aureus* (16.8 mm), *Bacillus subtilis* (18.3 mm), *Salmonella typhi* (14.5 mm), and *Escherichia coli* (17.2 mm) (Ashokkumar et al. 2015). The inoculation of *Ipomea carnea*-AgNPs on a cellulose acetate membrane exhibits a 14 mm inhibition zone against *Mycobacterium smegmatis* (Daniel et al. 2014). The AgNPs synthesized by *Boerhavia diffusa* show greater sensitivity on *Flavobacterium branchiophilum* compared with two other fish bacterial pathogens *Aeromonas hydrophila* and *Pseudomonas fluorescens* (Thakur et al. 2014). Lingo-berry- and cranberry juice-mediated AgNPs show a higher level of activity against *S. aureus*, *B. subtilis*, and *B. cereus*, but a low level of activity against *C. albicans* and food-borne *B. cereus* (Firdhouse and Lalitha 2015). The biosynthesized AgNPs using cell-free supernatants of *Staphylococcus aureus* exhibit significant antimicrobial activity against methicillin-resistant *S. aureus*, followed by methicillin-resistant *Staphylococcus epidermidis* and *Streptococcus pyogenes*, but with only moderate effects against *Salmonella typhi* and *Klebsiella pneumoniae* (Nanda and Saravanan 2009). The AgNP-mediated Broccoli floret aqueous extract are effective against human pathogens such as *Klebsiella pneumonia*, *Staphylococcus saprophyticus*, and *Escherichia coli* (Caroling et al. 2013). The AgNPs become attached to the surface of the cell membrane, disturb the function, and penetrate directly into the bacterial outer membrane and release the Ag ions (Caroling et al. 2013).

14.2.4 Anti-cancer Activities

The discovery and identification of a new antitumor drug with few side effects on the immune system has become major goal in many studies on immuno-pharmacology (Xu et al. 2009). The focus has increased towards developing potent anticancer and antitumor drugs based on the natural compounds from plants and marine bioresources and microorganisms (Devi et al. 2012). Most cytotoxic drugs act on cancer cell growth and division, but the co-application with nanomaterials could revolutionize cancer diagnosis and therapy (Abdullah et al. 2014; Gul-e-Saba and Abdullah 2015; Supraja et al. 2016; Hussein et al. 2020) and the encapsulation of therapeutic agents with NPs could improve targeted drug delivery systems (Abdullah et al. 2014). The use of metallic NPs and medical AgNPs has shown different degrees of in vitro cytotoxicity with the ability for passive or active targeting on any particular diseased cells or tumor tissues (Wicki et al. 2015). To overcome the limitations of conventional chemotherapy, the challenges will be to develop new NPs in single platform-based strategies and to address the physiological barriers, limited carrying

capacity, enhanced permeability and retention effect (EPR), the variability of NPs, and the regulatory and manufacturing issues (Wicki et al. 2015).

Although AgNPs may have low toxicity towards human cells with high thermal stability (El-Kassas and El-Sheekh 2014), the toxicity can be influenced by the availability of chemical, biochemical, and/or biological coatings on the NPs surface (Suresh et al. 2012). The surface charges of the AgNPs could determine the toxicity effects in the cells. The positive surface charge may make the cells more adaptable, allowing them to stay for a long time in the blood stream, as compared to the negatively-charged NPs (Tabata and Ikada 1988). This is pertinent for the regulation of anticancer agent (Tiyaboonchai 2003; Schlinkert et al. 2015). The AgNPs may interact with the thiol-rich enzymes, overlapping with the suitable functioning of the cellular proteins, and inducing changes in the cellular chemistry such as providing relatively high hydrophobicity inside the bovine hemoglobin, which causes a transition from alpha helices to beta sheets, leading to partial unfolding and the aggregation of protein (Shawkey et al. 2013; Supraja and Arumugam 2015). The anticancerous efficacies of the AgNPs synthesized through different sources have been evaluated against the Hep2 cell line (Devi et al. 2012; Rosarin et al. 2013), the HT-29 cell line, the Vero cell line, and breast cancer line MCF-7 (Devi and Bhimba 2012; Hussein et al. 2020). AgNPs synthesized using Acalypha indica Linn. exhibit only 40% cell inhibition toward human breast cancer cells (MDA-MB-231) (Krishnaraj et al. 2014). The viability of MCF-7 cells is also reduced to 50% at 5 μg/mL when treated with AgNPs biosynthesized by *Dendrophthoe falcata* (L.f) Ettingsh (Sathishkumar et al. 2014).

The AgNPs synthesized using *Aloe*, *Magnolia* leaves, and *Eucalyptus* leaves extracted at 2–4 ppm are found to be noncytotoxic to human embryonic kidney 293 cells, as analyzed by the automated InQ Plus equipment (Okafor et al. 2013). The stem latex of *Euphorbia nivulia*-capped AgNPs solubilize in water and act as a biocompatible vehicle for the transport of nanosilver to human lung carcinoma cells (A549) (Valodkar et al. 2011). No cytotoxicity effects of *Aloe vera*-conjugated AgNPs have been observed against human dermal fibroblasts (HDF) cells, but excellent antibacterial activity is reported against *E. coli* even at very low concentration (Zhang et al. 2010a, b). The *Chrysanthemum indicum*-AgNPs also exhibit no toxicity on 3T3 mouse embryo fibroblast cells at 25 μg/mL (Arokiyaraj et al. 2014). The AgNPs synthesized using *Origanum vulgare* exhibit a higher dose-dependent response toward human lung A549 cancer cell line (LD_{50} 100 μg/mL) (Sankar et al. 2013). The AgNPs biosynthesized using *Albizia adianthifolia* leaf extract at 10 and 50 μg/mL, show reduced viability of A549 cells to 21%, and 73%, and the normal peripheral lymphocytes to 117% and 109%, respectively, after 6 h exposure. This suggests that the AgNPs are potentially nontoxic to the normal healthy peripheral lymphocytes (PLs) (Gengan et al. 2013). However, the AgNPs synthesized using the root of *Morinda citrifolia* exhibit 100% cell death against the HeLa cell line at 100 μg of AgNPs (Suman et al. 2013).

The IC_{50} of A549 cells is at 43 μg/mL after AgNP treatment, which induces the cell death by ROS generation, resulting in apoptosis (Govender et al. 2013). The MCF-7 cells treated with *Sesbania grandiflora*-mediated AgNPs at 20 μg/mL, lead to nuclear condensation, cell shrinkage, and fragmentation after 48 h, with Hoechst staining. These changes confirm the activation of DNA repair due to the cleavage of the substrates (Jeyaraj et al. 2013). The Ag (protein–lipid)

nanoparticles (Ag-PL NPs) synthesized using *Sterculia foetida* (L.) seed extracts show cellular DNA fragmentation in HeLa cancer cell lines (Rajasekharreddy and Rani 2014). *Alternanthera sessilis*-mediated AgNPs at 25 µL/mL show complete apoptosis of about 95% against prostate cancer cells (PC3), whereas the growth of MCF-7 is inhibited almost 99% (Firdhouse and Lalitha 2013). *Datura inoxia*-AgNPs inhibit 50% of human MCF-7 proliferation at IC_{50} 20 µg/mL after 24 h incubation by inhibiting its growth, arresting the cell-cycle phases, and reducing the DNA synthesis, to induce apoptosis (Gajendran et al. 2014). The anticancer effects of starch-coated AgNPs have been studied in the normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251). The AgNPs show more sensitivity towards U251 cells than the IMR-90 cells by inducing changes in the cell morphology, reducing the cell viability and metabolic activity, and increasing the oxidative stress, leading to mitochondrial damage, increased ROS production, and DNA damage. The cellular uptake of the AgNPs occurs mainly through endocytosis, where the AgNP-treated cells show several abnormalities including upregulation of metallothionein, downregulation of major actin-binding protein, filamin, and mitotic arrest. The morphological changes of the cancer cells suggest that the AgNPs induce the cell death mechanism (Zhang et al. 2016). Figure 14.4 shows the mechanisms of AgNP-induced cytotoxicity in cancer cell-lines through endoplasmic reticulum stress (ER), lactate dehydrogenase (LDH), and ROS. Single-crystalline AgNPs have dose-dependent cytotoxic activity on the MCF-7 breast cancer cells through the induction of apoptosis, with 50% cell growth inhibition (LD_{50}) of 3.5 ng/mL and LD_{100} of 14 ng/mL (Franco-Molina et al. 2010). The ROS elevation caused by the AgNPs could damage the cell DNA as reported in some in vitro studies (Ahmad et al. 2008; Asharani et al. 2009; Foldbjerg et al. 2009).

The AgNPs have shown significant inhibitory effects on the activity of interleukin-5 (IL-5), interferon-γ (INF-γ), and tumor necrosis factor-α (TNF-α) (Shin et al. 2007). The AgNPs could destroy the tumor cells because of their plasmonic nature, where the light from the target cells can be absorbed and converted into thermal energy, leading to thermal ablation of the target cells (Loo et al. 2005; Nurani et al. 2015). The AgNPs may also stimulate cytotoxicity in phagocytosing cells in mouse peritoneal macrophages and human monocytes (Foldbjerg et al. 2009; Park et al. 2010; Shavandi et al. 2011). The Cytotoxicity activity induced through ROS leading to cell apoptosis, could be achieved at a lower AgNPs concentration and low incubation times (Braydich-Stolle et al. 2005; Carlson et al. 2008; Nishanth et al. 2011). The cytotoxic effects of AgNPs on MDA-MB-231 cells, resulting in the inhibition of the cell growth, the activation of LDH, increased level of ROS generation and the activation of caspase-3, are all essentials in the induction of apoptosis (Gurunathan et al. 2013a, b). The AgNPs biosynthesized from *Datura inoxia* extract exhibit anticancer activity after 24 h treatment, by inducing apoptosis in the MCF-7 cells via the ROS-mediated apoptotic pathway, leading to increased ROS levels, followed by the losses of mitochondrial membrane, leading to increased apoptotic morphological changes in the AgNP-treated cells. The DNA content is significantly reduced after staining with propidium iodide (PI), where the control cells exhibit very few PI-positive cells, while the treated cells show gradual increase in the number of PI-positive cells (Gajendran et al. 2014). The *Albizia adianthifolia*-based

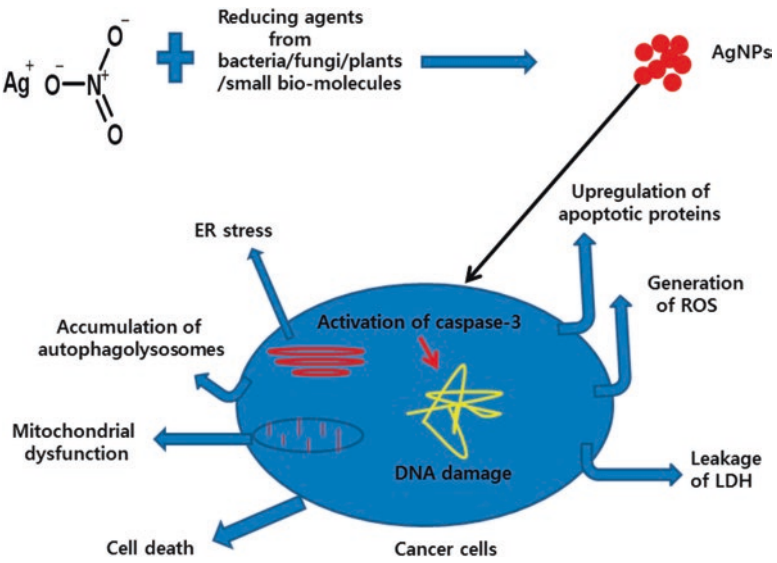

Fig. 14.4 The possible mechanisms of silver nanoparticle (AgNP)-induced cytotoxicity in cancer cell lines. Endoplasmic reticulum stress (ER), lactate dehydrogenase (LDH), reactive oxygen species (ROS) (Adapted from Zhang et al. 2016)

AgNPs have pro-apoptotic activities which activate the intrinsic apoptotic pathway in the lung carcinoma cells (A549) mediated by the CD95 death receptor. This induces the Fas-associated protein with death domain (FADD) adapter protein which binds to and activates caspase-8 through the formation of a death-inducing signaling complex, resulting in reduced CD95 expression and ATP concentrations. The increased level of lipid peroxidation as a result of ROS is also attributable to the disorders in the mitochondrial respiratory chain (Govender et al. 2013).

14.3 Conclusion

Nanoparticles and nanomaterials can be utilized for human medical applications including for the delivery of therapeutic drugs to cells, or for the imaging of tissues and organs. AgNPs have gained special interest for biomedical applications because of their SPR energy, which is located away from the inter-band transition energy, and their antioxidant, antimicrobial, and cytotoxic activities. The development of a reliable and environmentally friendly process for the synthesis of AgNPs is of great importance, especially with regard to meeting the economic and green production route. The mechanisms of AgNP-induced bacteria death include the destruction of membrane structure and permeability via the generation of many pits and gaps, resulting in the leakage of reducing sugars and proteins. The AgNP-induced cytotoxicity in cancer cell lines is through ER stress, LDH leakage, elevated ROS and finally the induction of apoptosis.

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Chapter 15
Nanomedicine for Ischemic Diseases: Recent Development and Future Challenges

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Abstract Ischemic diseases (CAD: coronary artery disease; CID: cerebral ischemic disease; PAD: peripheral artery disease and few others) are life-threatening problems and have become the most common cause of death worldwide. The conventional approaches (surgery, radiation therapy, chemotherapy, etc.) available in the market for the treatment and diagnosis of these diseases are associated with several limitations, including non-specificity to the targeted site, adverse effects to the surrounding normal tissues, systemic toxicity, high treatment cost, unavailability of facilities, etc. These situations have compelled the scientists to turn their heads toward the use of new technology in therapeutic and diagnosis of different ischemic diseases. Hence, emergence of nanotechnology has seen its utility in theranostic applications for ischemic diseases. In this book chapter, we aim to give an overview of the types and pathophysiology of ischemic diseases and ischemic reperfusion injury. Furthermore, in the upcoming sections, we have made detailed discussions about the treatment and diagnosis of ischemic diseases with the help of nanoparticle through active as well passive drug targeting. We have also concisely explained the current trends involved in the use of nanoparticle in the overall recovery of ischemic diseases. Finally, we have highlighted the challenges as well as the future perspectives of the potential use of the nanomedicine in the field of ischemic diseases.

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_15

Keywords Nanomedicine · Ischemia · Ischemic reperfusion injury · Nanotechnology · Drug delivery · Therapeutic · Diagnostics

15.1 Introduction

Ischemic diseases are considered to be the most prevailing cause of mortality and morbidity worldwide (Benjamin et al. 2017). The disease refers to a condition in which certain organs of the body get reduced supply of blood, oxygen, and nutrients mainly through the large arteries. The clinical symptoms are manifested due to narrowing of arteries or deposition of fatty plaques on the inner arterial walls (Ernst et al. 1987; Lee et al. 2018). This condition may damage organs reversibly or irreversibly. Depending upon the location of the body, ischemic diseases can be classified into three main types: coronary artery disease (CAD), peripheral artery disease (PAD) and cerebral ischemic disease (CID) (Kim et al. 2010; Lee et al. 2010; Rafieian-Kopaei et al. 2014). Some of the very common factors like obesity, lack of physical exercise, diabetes, hypertension, etc., majorly attribute to the disease (Goff Jr. et al. 2014). According to World Health Organization (WHO), coronary artery disease, also known as ischemic heart disease, claims the highest number of lives in European and Central Asian countries as compared to countries with high income (Finegold et al. 2013; Rao 2018). The statistical data recorded for the coronary artery disease accounts for nearly 45.1% deaths annually. In addition to the huge number of deaths, the high cost of its treatment also lays a significant socioeconomic burden. The cost estimated for the treatment of ischemic disease is reported to be 316 billion dollars in US (Bejarano et al. 2018). Another form of ischemic disease, which also records a considerably high rate of mortality (87%) per year, is CID, also called ischemic stroke (Kaviarasi et al. 2019).

The conventional treatment options generally recommended include diet therapy, medicinal therapy, surgery, etc. (Giacoppo et al. 2016; Lloyd-Jones et al. 2016; Reiner et al. 2016; Behzadi et al. 2017; Robertson et al. 2017). These procedures pose harmful effects on individuals. Thus, there is an urgent need to look beyond the conventional methods and conceive safer and cost-effective treatment options for both diagnostic and therapeutic purposes. Since the last decade, nanotechnology has slowly and steadily paved its way into the field of medicine, by providing excellent therapeutic and diagnostic applications and also by forming an important integrant of various medicines (Mukherjee et al. 2015; Behzadi et al. 2017; Jiang et al. 2017; Karahan et al. 2018).

Therefore, scientists and researchers, including our group, have started to employ nanotechnology in the treatment and diagnosis of ischemic diseases (Zhang et al. 2017b; Wang et al. 2018; Vemuri et al. 2019). In this chapter, we have focused on the types, pathophysiology, and reperfusion injury of mainly three types of ischemic diseases, namely, PAD, CAD, and CID. We have discussed how the advent of nanotechnology has influenced the methods of treatment and diagnosis of different

ischemic diseases through various modes. Finally, the chapter is concluded by shedding light on the future perspectives and challenges on the use of nanoparticles in ischemic diseases.

15.2 Ischemic Diseases and Their Pathophysiology

15.2.1 Types of Ischemic Diseases

Depending upon the portion of the body affected, ischemia can be referred to as (1) peripheral ischemia, also known as peripheral artery disease (PAD) when the organs of extremities like limbs are affected; (2) coronary artery disease (CAD), when coronary arteries are blocked and the heart gets affected; and (3) cerebral ischemic disease (CID), when the brain gets affected.

15.2.1.1 Coronary Artery Diseases (CAD)

Coronary artery disease (CAD) is defined as the condition of the heart where there is a lack of supply of blood, oxygen, and nutrients in the heart muscles (Tham et al. 2015). This condition damages the heart reversibly or irreversibly and generates heart diseases. The blockage in the coronary arteries is a result of formation of atherosclerotic plaque within their lumen. The pathological condition gains more prevalence with the increase in the age of individuals. Often, complete blockage of the blood supply to the heart leads to fatal conditions. General factors responsible for the disease include sedentary and stressful lifestyle, unhealthy food habits, alcoholism, smoking, diabetes mellitus, hypertension, hyperglycemia, and many more (Fowkes et al. 2017). According to World Health Organization, CAD claims the largest number of deaths worldwide (Benjamin et al. 2019). Common remedial strategies employed for the prevention of the disease include lipid-lowering therapies, diet therapy, etc. Apart from them, the treatment options for severe conditions preclude surgical procedures like angioplasty, coronary artery bypass grafting, stent placement inside arteries, etc. Although these modalities are effective, they have various shortcomings like invasiveness, systemic toxicity with medication or stents (Binsalamah et al. 2012; Rabito and Kaye 2013), high treatment cost, and finally prolonged treatment period.

15.2.1.2 Peripheral Artery Disease (PAD)

Peripheral arterial disease (PAD) is caused by the obstruction of blood supply to the lower extremities of the body through non-myocardial arteries (Tang et al. 2012). It is one of the most prevalent vascular diseases in the world. The pathological

symptoms that are manifested occur as a result of lack of supply of oxygen and nutrients to the organs affected. This condition often makes the affected region of the body incompetent, thus adversely affecting the quality of life of the patients diagnosed with the disease. PAD exhibits a wide spectrum of symptoms such as claudication, ulcers, rest pain, skin lesions like ulcers or gangrene, etc. (Abu Dabrh et al. 2016). The most common form of PAD is limb ischemia, where the limb gets affected (Berger Jeffrey and Hiatt William 2012). Lack of early diagnosis can lead to complete blockage of blood supply to the limbs, resulting in a serious condition called critical limb ischemia (Dua and Lee 2016). It has an extremely high rate of mortality and morbidity. Reinstitution of blood supply to the limbs is one of the reliable therapies for treatment, but in many cases of critical limb ischemia (CLI), amputation seems to be the only option for treatment. Conventional therapies that are generally recommended as a means to combat the symptoms include angioplasty, bypass surgery, atherectomy, stent implantation, etc. (Giacoppo et al. 2016; Olin et al. 2016; Patel et al. 2016; Strom et al. 2016; Robertson et al. 2017; Wabnitz and Turan 2017).

15.2.1.3 Cerebral Ischemic Disease (CID)

Brain ischemia is defined as a disease that arises due to deprivation of blood to the brain to perform regular metabolic activities. This results in decreased oxygen supply to the brain, also known as cerebral hypoxia, which causes cerebral ischemic stroke (White et al. 2000; Lee et al. 2018) . The ischemia can be classified into two types, namely, focal ischemia and global ischemia depending upon how much area of brain it affects. Focal brain ischemia affects a confined region and is caused when a cerebral vessel is obstructed by a blood clot. This results in a decrease in blood flow to a particular region and causes death of the cells in that region (Miettinen et al. 1997). Global ischemia, on the other hand, is caused by decreased blood supply to the brain, which is mainly caused by cardiac arrest (Auer 2016). The types of cerebral ischemia include thrombotic ischemia, in which the blood vessel is blocked due to a clot or arterial spasm and embolic ischemia, that owes to formation of blood clot in the artery, which then goes to a different artery to make a new blockage there. The last type of ischemia is hypoperfusion, which occurs due to less amount of blood supply that may be due to heart attack, trauma, etc. (Lee et al. 2018). Stroke or ischemic stroke is considered as a type of cerebral ischemia and is a major cause of unhealthy mind and death. The stroke that is caused by ischemia accounts for 87% (Ovbiagele and Nguyen-Huynh 2011). Ischemic stroke is caused by the development of vascular thrombus and blockage in the blood supply to the brain, resulting in deficiency of neurological activities and death of neuronal cells (Janardhan and Qureshi 2004; Vijayan and Reddy 2016). The ischemic stroke mainly occurs at the site of middle cerebral artery, which is considered to be the relatively greatest of all branches of the internal carotid artery. This artery mainly deals with the supply of oxygen and different nutrients to the brain (Navarro-Orozco and Sánchez-Manso 2018).

15.2.2 Pathophysiology of Ischemic Diseases

15.2.2.1 Coronary Artery Disease

Based on the estimated number of mortalities worldwide, CAD can be called a fatal disease. Despite its inevitable nature, if it is diagnosed early, it is a highly predictive, preventable, and treatable disease. Apart from the conventional risk factors that have been mentioned earlier in this chapter, other underlying mechanisms also constitute more than 40% of prime cause of the disease. These risk factors include high atherosclerosis, oxidative stress, low antioxidants, chromosomal aberrations due to hypertension, hyperlipidemia, and diabetes mellitus (Simon and Vijayakumar 2013). Among these, atherosclerosis is the leading cause of both CAD and PAD. Atherosclerosis is associated with thrombosis and inflammation and the lesions caused by it mainly contain smooth muscle cells, macrophages, and lipids. In patients with hypercholesterolemia, excess low density lipoprotein (LDL) in the blood accumulates in the arterial lumen, majorly at sites where the blood vessels bifurcate and blood flow is disturbed (Rafieian-Kopaei et al. 2014). These LDLs first accumulate on the inner walls of arteries at bends and then infiltrate the arteriolar wall to reach the intima. Inside the intima, they become oxidized. The oxidized LDLs lead to the release of phospholipids that stimulate the overlying endothelial cells to express adhesion molecules and inflammatory molecule like macrophage stimulating factor (M-CSF). Among the adhesion molecules, VCAM-1, MCP-1, E-selectins are most prevalent and they recruit the monocytes and lymphocytes that express VLA-4, CCR-2, L-selectins (Hwang et al. 1997; Lusis 2000; Silva et al. 2018). These monocytes then enter the tunica intima, where they engulf the oxidized LDL via scavenger receptors and toll-like receptors. The oxidized LDLs are destroyed inside the macrophages and the cholesterol exposed from them is retained as cytosolic lipid droplets inside the macrophages. These macrophages are then termed as foam cells. The activated macrophages produce inflammatory cytokines, chemokines, proteases, and radical molecules. The activated macrophages stimulate the T-cells that in turn produce interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukins-1. All these factors promote inflammation in atherosclerosis. Simultaneously, regulatory T-cells produce interleukin-10, transforming growth factor-β (TGF-β) as protective factors against atherosclerosis (Hansson 2005). As a consequence of inflammation, SMCs also proliferate and migrate to the tunica intima and form a thick covering called the fibrous cap over the plaque. This fibrous cap also consists of T-lymphocytes, macrophages, collagen-rich fibrous tissues. These SMCs, along with endothelial cells and monocytes, secrete matrix metalloproteinases (MMPs) that also promote proliferation of cells, apoptosis, destruction of extracellular matrix of arteries, and inflammation. Apoptosis of foam cells leads to the release of lipids from inside them. These lipids accumulate inside the atherosclerotic lesion and form the necrotic core (Libby and Theroux 2005; Yu et al. 2011). This increases the volume of the atherosclerotic plaque, and as such it intrudes the lumen of the arteries. On the other hand, the MMPs and also

metaproteinase secreted by macrophages that have the ability to destroy the extracellular matrix can destroy the fibrous cap also. This rupture of fibrous cap exposes the collagen and lipids to blood stream, which contributes to accumulation and adhesion of platelets and blood clot formation that ultimately blocks the blood stream (Rafieian-Kopaei et al. 2014).

15.2.2.2 Peripheral Artery Disease

PAD usually implicates atherosclerosis and thrombosis in the arteries of lower part of the body like abdominal aorta, iliac and femoral arteries, leading to end organ ischemia. Athero-thrombosis is a complex interplay of a large number of cells, proteins, and pathways along with blood cholesterol and vascular cells. An increased level of LDL cholesterol in the blood passes through the endothelial cells of the blood and reaches tunica intima and gets oxidized. This prompts the ECs to express pro-inflammatory molecules like chemokine adhesion molecules and growth factors (M-CSF) on their surfaces. This pro-inflammatory molecule attracts the monocytes and lymphocytes to this site. These monocytes then enter into the tunica intima and differentiate into macrophages. The oxidized LDL secretes cytokines that activate the macrophages to phagocytose the oxidized LDL and form foam cells. This foam cell accumulation appears as fatty streak. When the foam cells die, their lipid content sare released into the tunica intima forming a lipid core. This forms the atherosclerotic plaque. Within this lipid core, other substances like cytokines released by monocytes, TNF, growth factors, precoagulation substances, calcium ions, etc., also accumulate. Simultaneously, the SMCs that migrated into the tunica intima also proliferate and form a fibrous cap overlying the lipid core. This lesion, i.e., lipid core, continues to grow and eventually starts blocking the lumen of blood vessels. Thus, blood perfusion to distal targets is blocked (Burnett 2004; Muller et al. 2013; Rafieian-Kopaei et al. 2014).

15.2.2.3 Cerebral Ischemic Disease

There have been many reports for the pathophysiology of cerebral ischemic stroke. In one study, Deb et al. (2010) mentioned that after the occurrence of cerebral ischemia there can be observed a decreased amount of oxidative phosphorylation of ATP due to interruption in the supply of oxygen and nutrients mainly glucose. The pH of the brain tissue also lowers due to an increase in anaerobic glycolysis (Hagihara et al. 2018). The decrease in the ATP results in a series of events like inability in maintaining cell homeostasis, loss of neurons and glial membrane potential, and subsequent depolarization with the invasion of Ca^{2+} into cell (Bylicky et al. 2018). The phase of depolarization is followed by the release of amino acids of excitatory nature into the extracellular space and disruption in the re-intake of excitatory amino acids by the presynaptic amino acids. This results in the

aggregation of glutamate and Ca^{2+} (Kyle and Saha 2014). All this results, in the destruction of mitochondrial and cell structures due to ROS generation. This usually occurs after reperfusion (Da Silva-Candal et al. 2017). Many intracellular signaling pathways are also generated, and this cumulatively causes apoptosis and necrosis of cell (Kunz et al. 2010). These events are classified accordingly as excitotoxicity, inflammation, and oxidative stress. These events have a direct effect on the blood brain barrier (BBB). It has been known that BBB is formed of brain endothelial cells (BEC) and helps in maintaining fluids, nutrients, cells, and brain homeostasis (Jiang et al. 2018). The ischemic stroke and reperfusion have a negative effect on the BEC, which results in the destruction of their tight junctions. This, in turn, affects BBB by increasing its permeability to all substances and its dysfunction (Jiang et al. 2018).

15.3 Nanotechnology in Biological Application

Nanotechnology, in the recent years, has proved to be an excellent technology for varying applications in every field of life. The variability in the properties of the nanoparticles has allowed it to find its applications in different fields such as electronics, space research, physics, computer science, chemistry, agriculture, food, cell biology, medicinal applications, pharmaceuticals, and many more (Heiligtag and Niederberger 2013; Krol et al. 2013; Kandil 2016; Jeevanandam et al. 2018; He et al. 2019; Prasad et al. 2018; Thangadurai et al. 2020). Nanotechnology involves a multidisciplinary area, including designing, synthesis, and characterization of nanoparticles. Nanotechnology has slowly and steadily paved its way into medicinal applications since, many a time the commercial products available in the market are unable to provide the required therapeutic or diagnostic effect needed for the treatment. Because of the shapes and sizes of the nanoparticles, they can interact with different proteins, nucleic acids, and a variety of macromolecules present in the body (Bejarano et al. 2018). Nanoparticles have a size range of 1–100 nm that are often biocompatible and can easily enter into the cell or their organelles, without inducing toxicity. Nanoparticles have the ability to stimulate different target moieties, show response, and control various processes with increased sensitivity and reactivity (Bamrungsap et al. 2012; Rizvi and Saleh 2018). For example, nanoparticles can be used as a delivery vehicle loaded with therapeutic agents that can target diseased regions and release their contents in sustained manner. Other applications of nanoparticles include defined surface area, increased reactivity area, surface plasmon resonance, and ability to change structure and morphology such that they can act as an alternative treatment in different diseases (Wang et al. 2016; Khan et al. 2017). Even functionalized nanoparticles or nanocomposites are able to render various multifunctional applications like therapeutics and diagnostic applications. (Anselmo and Mitragotri 2016). Therefore, nanotechnology is used for diagnostics and therapeutics and in imaging of various diseases (ischemic, cancer, diabetes) (Mukherjee et al. 2012; Di Santo et al. 2015; Ojha and Kumar 2018).

15.4 Nanoparticles for Diagnosis of Ischemia

15.4.1 Diagnosis of CAD

Despite immense therapeutic advancements, coronary artery disease remains one of the leading causes of death globally. Approximately, 16 million people are affected by the disease and an estimated 325,000 people die off cardiac arrest annually. The most common factor responsible for cardiac arrest involves the rupture of atherosclerotic plaques and subsequent blockage of coronary arteries, leading to heart failure (Yu et al. 2011; Rafieian-Kopaei et al. 2014). Prior recognition of the development of the disease is pivotal to properly buffer the manifestation of the symptoms. Prevailing conventional strategies for diagnosing the disease are unable to diagnose the disease at its inception. New diagnostic tools and procedures need to be introduced in this domain in order to overcome the shortcomings of conventional therapies. Nanotechnology already offers a number of avenues for improvising the therapeutic aspect of the disease. Hence, it can also be employed to distinguish the vulnerable plaques in atherosclerosis. With nanomedicine, we can develop strategies based on targeting the molecular markers responsible for the development of the disease. The ability of the macrophages to phagocytose nanomaterials can be used as contrast agents to trace the formation of atherosclerotic lesions. Even biomarkers for inflammatory molecules involved in the progress of the disease can be targeted by nanomaterials to produce the tracking technique (Schoenhagen and Conyers 2008; Zhang et al. 2018).

The vulnerable atherosclerotic plaques in CAD contain lipid-laden macrophages or rather foam cells that undergo both apoptosis and necrosis (Martinet et al. 2011). Tracing these apoptotic and necrotic plaques through imaging techniques can help reduce the risk of disease development. It is known that during apoptosis, the phosphatidylserines (PS) of the cell membranes are redistributed by flippase enzymes from the inner layer to the outer layer. Annexin A5 (AnxA5), which has high affinity for these PS, is used to detect apoptotic cells. These annexin A5 molecules are often radiolabelled to detect apoptotic cells. In this context, De Saint-Hubert et al. has made several attempts to detect PS in apoptotic cells using several different forms of radionuclides (123I, 124I, 99mTc, and 18F) for diagnosis of coronary diseases. Finally, the author and his group observed that 99mTc radionuclide labeled annexin A5, when attached with fluorescent probes, could successfully locate and produce images of apoptotic cells in SPECT-CT in APO−/− knockout mice. Similarly, the team also established that Iodine-124 (I124) radiolabeled annexin A5 can detect and produce PET images of apoptotic cells in coronary artery disease (De Saint-Hubert et al. 2014).

The progression of inflammation of atherosclerotic plaques can be targeted for visualization by in vivo imaging tools. Flögel et al. (2008) used nanoparticles containing perfluorocarbons (PFCs) for use in MRI imaging studies. The PFC used here

was perfluoro-15-crown-5 ether, which has all 20 fluorine nuclei that are equivalent both chemically and magnetically and hence suitable for ^{19}F MRI detection (Dardzinski and Sotak 1994; Partlow et al. 2007). The author and his group induced cardiac infarction in murine models by ligation of LAD. The administration of PFC was done intravenously. 19F MRI imaging showed the existence of PFC near the infarcted region. FACS analysis revealed that the fluorescence-labeled PFCs were found to be taken up by macrophage monocytic cells, exhibiting marker CD11b (Flögel et al. 2008).

15.4.2 Diagnosis of PAD

The advancement of nanotechnology has revolutionized the mode of molecular imaging and characterization of disease pathophysiology (Chakraborty et al. 2011). Early diagnosis and prompt treatment of a disease like PAD can evade the need for prolonged treatment and even amputation. Conventional diagnostic techniques include angiography, ultrasonography (USG), computed tomography angiography (CTA), magnetic resonance angiography (MRA), etc. Nanomedicine offers safe and proficient system for delivery of cargo, and this efficiency can be utilized to circulate imaging agents, fluorescent tags, and other biologically important devices, which can be detected by modalities including magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), computed tomography (CT), optical Near Infrared fluoroscopy (NIRF), etc. at target sites (Zhang et al. 2017b). The imaging agent or fluorescent-tagged nanoparticles can give us information about many aspects of the disease, most importantly about the location and status of the pathophysiology of the disease. Targeting of biomarkers for assessing diseases by antibody and fluorescent-coated nanoparticles can result in more accurate detection of diseases. These biomarkers may be cell surface antigens, secretory molecules, etc.; and their expressions vary distinctly during different diseases or different states of the disease. In the context of diagnosis of ischemia or any vascular disease, it is a common practice to target vascular endothelial cell-specific markers like integrin, cell adhesion molecule (CAM), intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), platelet endothelial cell adhesion molecule (PECAM) etc. (Brown et al. 2008). Integrins are cell surface adhesion molecules expressed on the surface of endothelium. An increased amount of integrin expression during hypoxia can give a clue about vascular disease. Integrins like $\alpha_v\beta_3$ are expressed increasingly on vascular endothelial cell and surfaces can give us an idea about the condition of vascularization. These integrins can be targeted to evaluate the condition of PAD. When $\alpha_v\beta_3$ integrin-targeted PFC nanoparticles are administered in rabbits induced with limb

ischemia, the nanoparticles produced signals in MRI and X-ray angiography. The minor differences of angiogenesis levels in different treated animals were also observable as differences in signal strengths. The results of MRI and X-ray angiography corroborated with the results of histopathology of muscle tissues, which showed an improvement in vascularization upon treatment with pro-angiogenic molecules (Winter et al. 2010). During hypoxic conditions in the ischemic tissues, there is an upsurge in the secretion of growth factors to facilitate neovascularization by stimulating the mobilization of endothelial cells of existing blood vessels. All these mechanisms make the blood vessels leaky, and this loophole is a therapeutic target for curative as well as imaging purposes for ischemia. Kim et al. (2010) invented an imaging system for imaging of murine hindlimb ischemic muscle. The author formulated a PEGylated silica nanoparticle doped with Rhodamine or Cys-5 fluorescent tags. The PEG coating allowed the nanoparticle to circumvent the RES encapsulation and stay in blood circulation for longer time, while the fluorescent tags enabled to trace the nanoparticles at the ischemic tissues compared to the non-PEGylated nanoparticles (Kim et al. 2010). Sun et al. (2013), in his studies, reported the recent advances in nanotheranostics for the PAD. They designed a multifunctional VEGF-loaded IR800 conjugated GO nanoparticles (GO-IR800-VEGF) for imaging-based therapeutic angiogenesis in murine hindlimb ischemia. NIR fluorescence imaging revealed that the nanoformulation accumulated in the ischemic muscle tissues. Ex vivo immunostaining with CD-31 and α-SMA antibodies corroborated with the results of LDPI, PET, PA that showed therapeutic angiogenesis. The clearance efficacy of the nanoparticles was confirmed by strong NIR fluorescent signals in kidneys and bladders. Thus, the author sheds light on the applicability of GO for theranostic purposes for treating ischemic diseases (Sun et al. 2013).

15.4.3 Diagnosis of CID

There have been major backdrops of using many macromolecular substances, drugs, proteins, and nucleic acids for diagnosis due to their decreased permeability through blood brain barrier (BBB) (Hawkins and Davis 2005; Haddad-Tovolli et al. 2017). Hence, there have been large requirements for proper diagnostic tools for ischemic stroke. There are various imaging methods available at present for ischemic stroke such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission spectroscopy (PET), and single photo emission computed tomography (SPECT) (Pitek et al. 2017). But again these techniques have their own disadvantages because sometimes they produce distorted pictures and get hindered by biological components that make image formation difficult. With the emergence of nanotechnology, it has been a boon to use them as they act as enhancers that help to take better image of the ischemic diseased site (Provenzale and Silva 2009). There has been a lot of research going in the field of nanotechnology to use nanoparticles

as a means of diagnostic tools so that it can cross the major obstacle of the blood brain barrier (Zhou et al. 2018).

Magnetic resonance imaging (MRI) is a technique for imaging the brain in the absence of ionizing radiations. It helps to visualize the brain and helps in distinguishing the normal brain site from the diseased one (Ohki et al. 2019). MRI is used to receive information about the diseased site, but in some cases, the signals get interfered in some patients by the cardiac pacemakers or some metallic instruments that have been used in the patient's body previously for some issues in the magnetic field (Götte et al. 2010). The MRI sometimes also catches NMR signals coming from the water protons that are either bound to the tissues or in free form to put up an image of the tissue by recognizing contrast signals coming from free water and bound water. But sometimes the images are less clear due to problem in detecting intensities (Fink and Fink 2013).

Therefore, to overcome the limitations of the MRI imaging, several nanoparticles are being used that have paramagnetic properties and are used as enhancers. The nanoparticles increase the intensities of the bound water by changing their relaxation times, which in turn help in acquiring better images. There are again two types of contrast agents, positive and negative, depending upon the illumination they provide (Myerson et al. 2011). Some examples of enhancers include gadolinium, which acts as a contrast enhancer whereas iron oxide act as negative enhancers in MRI (Bogdanov Jr. and Mazzanti 2011).

Recently, Wang et al. (2018) demonstrated the utilization of iron oxide nanoparticles as MRI contrast agents of the collateral vessels, where the nanoparticles were decorated with RGD peptide. Here the peptide can interact with the αvβ3 integrins, which helps in the nanoparticle localization that can be viewed by the MRI imaging (Wang et al. 2018). Gao et al. (2011) discovered a very innovative nanoparticle for MRI imaging in ischemic cases, where nanoparticles are pH sensitive. They have coated iron oxide nanoparticles with poly(β-amino ester)-poly(amidoamine)-poly(ethyleneglycol) and the particles formed micelles. So when they are injected into the ischemic rats, the iron oxide would come out in the acidic condition of the ischemic area and helps in the recognition of the ischemic area during the MRI imaging (Gao et al. 2011). Other than this, Brisset et al. (2018) used to check the macrophages involved in the neuroinflammation, which causes stroke-related destruction to the brain at the ischemic region through small iron oxide nanoparticles that have supermagnetic properties. This helps in easy recognition through MRI (Brisset et al. 2018).

In the case of multimodal imaging involving a combination technique of CT scan and NIR, Hoehn et al. (2002) used HT22 hippocampal cells which expressed GFP and was labeled with iron oxide particles having superparamangnetic properties helped in the MRI imaging of the ischemic region of the brain. The cells were implanted into the ischemic site of the rats to view through MRI imaging. In another experiment, Xu et al. (2017) covered trimodal contrast substances including ink for the purpose of photoacoustic imaging MRI done with iron oxide nanoparticles, and ultrasonography was performed using perfluorohexane with PLGA as can be seen

in Fig. 15.1. All these were used to image the blood clot in the thrombus rabbit model where these targeted EWVDV (Glu-Trp-Val-Asp-Val) peptides by the use of P-selectin. The combinatorial approach helped in better imaging of the diseased site (Xu et al. 2017).

The positron emission tomography (PET) scan is used for the identification of the markers and the physiological components of the ischemic region, especially the penumbra area where the functionality is affected, not the morphological features. This technique is sometime being used in combination with MRI and CT scan. Like Glaus et al. (2010) used iron oxide nanoparticles exhibiting superparamagnetic properties that had a labeling of ^{64}Cu which can be used both for MRI and PET imaging. In another experiment, Keliher et al. (2017) used polyglucose nanoparticles with ^{18}F modification, which was used for both MRI and PET imaging in animals with cerebral ischemia.

Ultrasound is used for the detection of blockage in the blood vessels or formation of any blood clot. The whole mechanism is based on the property of echogenecity, where the ultrasound of high frequency is used and the reflections coming from the diseased area is recorded. There have been some problems with the reflection of these ultrasounds from the blood vessels; hence; contrast enhancers are being used for proper ultrasound imaging. Many scientists have started using nanoparticles as enhancers for the ultrasound imaging of ischemic diseases. Chen et al. (2013) used perfluorocarbon emulsions whose surface is modified with antibodies and helps in the ultrasound imaging of cerebral ischemic region in the thrombus by targeting fibrin. Nowadays, photoacoustic imaging is also used for ultrasound imaging. In this context, Cui et al. (2017) used perylene tetra carboxylic diimide (PDI) nanoparticles considered as semi-conductors are modified on the surface of cyclic RGd and is used for blood clot photoacousting imaging by targeting thrombus (Cui et al. 2017).

Hence, from the above studies, authors claimed that nanoparticles could be used as a therapeutic and diagnostic system for imaging. As it is depicted in the Fig. 15.2, the nanoparticle can be conjugated with multiple ligands and therapeutic moieties to cross the BBB (Kaviarasi et al. 2019). Table 15.1 depicts the diagnostic properties of nanoparticles in the diagnosis of ischemic diseases.

15.5 Nanoparticles in the Treatment of Ischemic Diseases

In this section, we focus on the nanotechnology-based advancements and strategies to cure ischemic diseases (CAD, PAD, CID).

Fig. 15.1 (continued) images of EWVDV–Fe–Ink NPs, EWVDV–Fe–PFH NPs, and EWVDV–Fe–Ink–PFH NPs after LIFU irradiation in the B mode and contrast mode. (**g**) Acoustic intensities of the EWVDV–Fe–Ink NPs, EWVDV–Fe–PFH NPs, and EWVDV–Fe–Ink–PFH NPs after LIFU irradiation ($*P < 0.05$, $***P < 0.001$) (Reprinted with permission from Xu et al. (2017) Phase transition nanoparticles as multimodality contrast agents for the detection of thrombi and for targeting thrombolysis: in vitro and in vivo experiments. ACS Applied Materials and Interfaces 9:42525–42,535. Copyright © (2017) American Chemical Society)

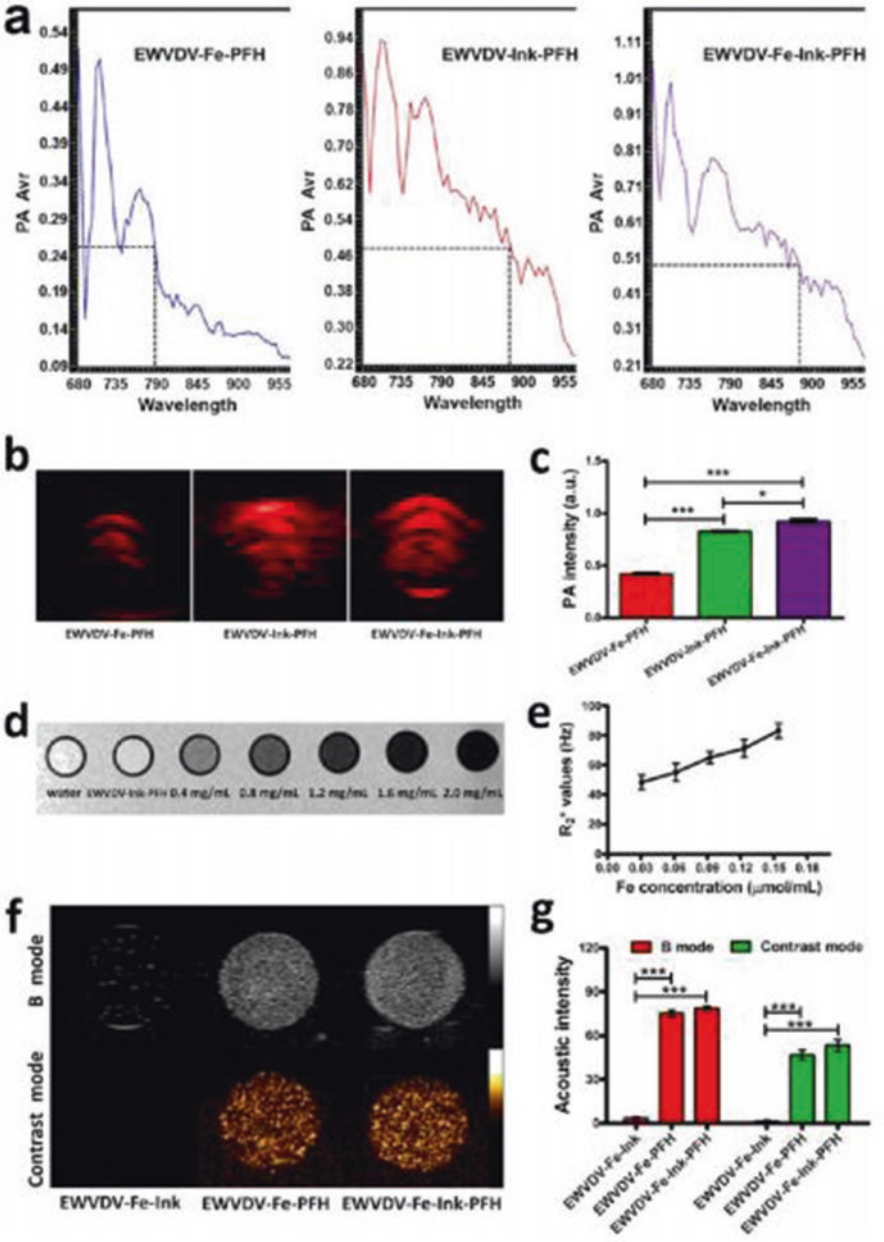

Fig. 15.1 Multimodal property testing of the NPs in vitro. (**a**) Absorption range of the PA intensities of the EWVDV–Fe–PFH NPs, EWVDV–Ink–PFH NPs, and EWVDV–Fe–Ink–PFH NPs by full-wavelength scanning (680–960 nm). (**b**) PA images (wavelengths of the Ink NPs and non-Ink NPs: 706 and 710 nm, respectively, laser fluence: 2 mJ/cm^2). (**c**) PA intensities of the EWVDV–Fe–PFH NPs, EWVDV–Ink–PFH NPs, and EWVDV–Fe–Ink–PFH NPs. (**d**) T2∗-weighted MR images of water, EWVDV–Ink–PFH NPs, and EWVDV–Fe–Ink–PFH NPs at different concentrations. (**e**) Validation curves of the $R2*$ values of the Fe NPs at different concentrations. (**f**) US

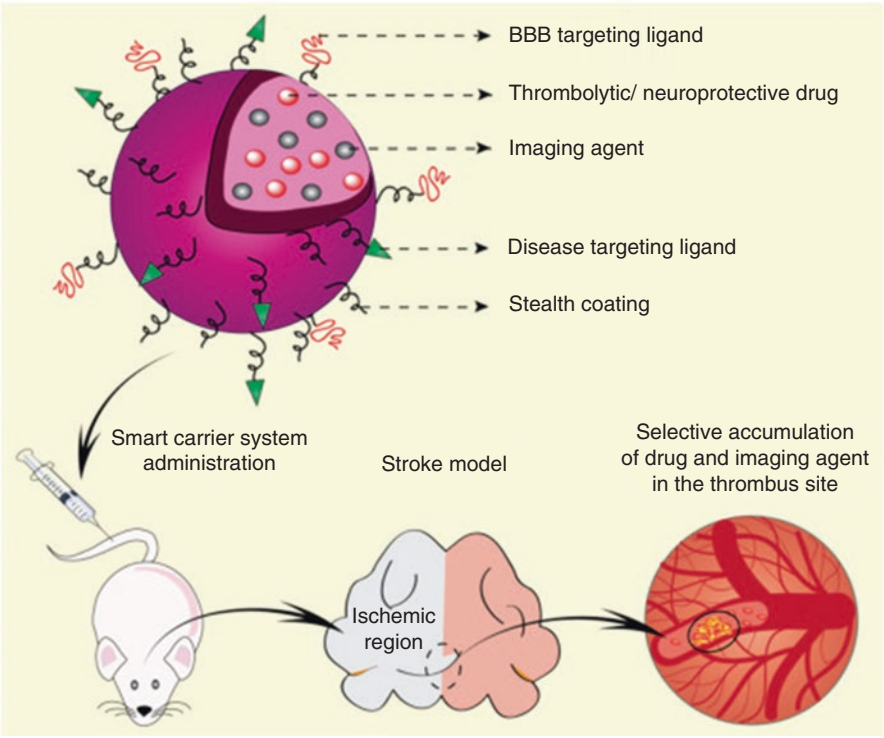

Fig. 15.2 Schematic representation of a 'smart nanotheranostic system' for the treatment of cerebral ischemia. Dual targeted carrier with therapeutic agent and imaging agent administration enhances accumulation of drug and imaging agent in the ischemic region. Therapeutic outcome of the treatment will be monitored with the help of imaging agent (Reproduced with permission from Kaviarasi et al. (2019) Emerging paradigms in nanotechnology for imaging and treatment of cerebral ischemia. Journal of Control Release 300:22–45. Copyright © (2019) Elsevier)

15.5.1 Treatment of CAD

The field of nanotechnology and nanomedicine is ever expanding. Many exciting innovations are coming up, all of which are ultimately aimed at inventing curative measures for different diseases. In this regard, CAD, which is one of the major causes of death of more than 30% of the people above the age 35, is also given due consideration (Mozaffarian et al. 2016). CAD describes a disease process in which atherosclerotic plaque accumulates on the endothelial lining of the coronary arteries and reduces the diameter of the arterial lumen. This causes reduced supply of blood to the heart. This in turn can damage the myocardium of the heart reversibly or irreversibly, based on the severity of the condition (Brito and Amiji 2007).

Proper functioning of coronary arteries largely depends of the endothelial lining. An injury in the endothelium can result in the formation of atherosclerotic plaque and that in turn can result in coronary artery occlusion. A prevalent way of treating

Table 15.1 Nanoparticles for diagnosis of ischemic diseases

Nanoparticles	Diagnostic approach	References
Perfluorocarbon (PFC) nanoparticles	$\alpha_v\beta_3$ integrin targeted and produce signals in MRI and X-ray angiography for limb ischemia	Winter et al. (2010)
PEGylated silica nanoparticle	Rhodamine or Cys-5 fluorescent tags helps in imaging purpose for hindlimb ischemic muscle	Kim et al. (2010)
Graphene oxide (GO) nanoparticles	VEGF loaded IR800 conjugated and helps in imaging-based therapeutic angiogenesis in murine hindlimb ischemia	Sun et al. (2013)
Perfluorocarbon (PFC) nanoparticles	MRI imaging near infracted region	Dardzinski and Sotak (1994); Partlow et al. (2007)
Iron oxide nanoparticles	RGD peptide conjugated and MRI imaging of collateral vessels	Wang et al. (2018)
Iron oxide nanoparticles	poly(β-amino ester)-poly(amidoamine)-poly(ethyleneglycol) MRI imaging of cerebral ischemic area	Gao et al. (2011)
Iron oxide nanoparticles (combinatorial approach)	MRI and ultrasonography for cerebral ischemia	Xu et al. (2017)
Iron oxide nanoparticles	^{64}Cu conjugated and MRI and PET imaging for cerebral ischemia	Glaus et al. (2010)
Polyglucose nanoparticles	^{18}F modification and MRI and PET imaging for cerebral ischemia	Keliher et al. (2017)
Perylenetetracarboxilicdiimide (PDI) nanoparticles	Cyclic RGD blood clot photoacousting imaging	Cui et al. (2017)

this type of coronary artery occlusion is to implant a stent in the affected region so as to keep the artery inflated. However, this treatment method is also afflicted with limitations like impaired endothelization at the concerned site. Irregularity in the healing of the endothelium at the coronary intervention site does not help revert coronary artery disease in the long term. Hence, successful cardiovascular therapy should preclude promotion of growth of endothelium at the target site. In this regard, coating of stents used for coronary intervention with bioactive agents that promote recovery and growth of endothelium is advisable. Ceylan et al. (2011) introduced this approach in his study on coronary artery disease. The self-assembled peptide biocompatible, amphiphiles Dopa (3,4-dihydroxyphenyl-L-alanine) helped in nanofiber coating on the stainless steel and REDV (Arg-Glu-Asp-Val), promoted adhesion, viability, and proliferation of endothelial cells over smooth muscle cells and platelets at the target site. The main cause for the enhancement of growth of the endothelium is that the REDV-PA offers a microenvironment that mimics the native extracellular matrix needed for proper growth of endothelial cells. Successful efficacy of this strategy was proven by positive in vitro results. Fluorescently labeled

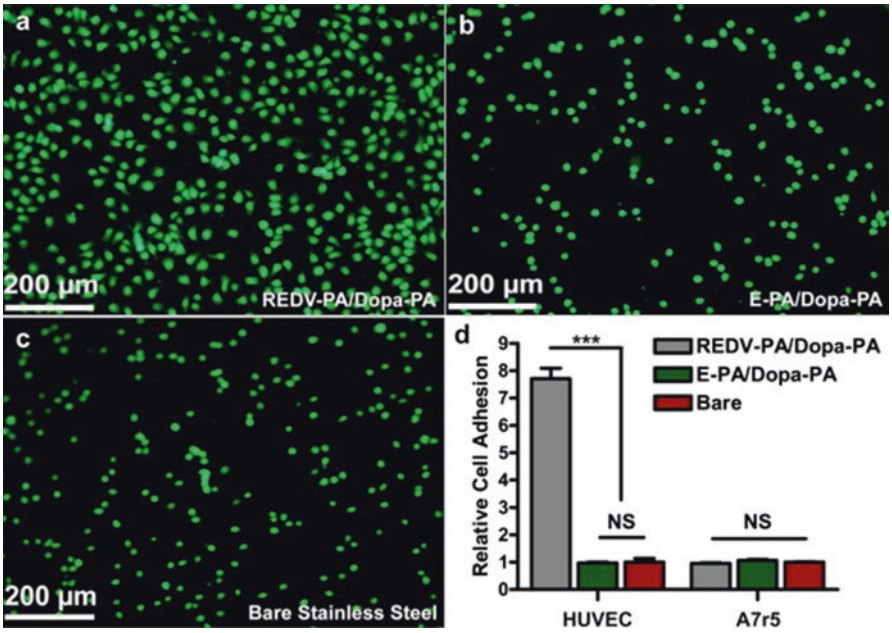

Fig. 15.3 Representative Calcein AM stained fluorescent images of HUVECs adhered on the stainless steel surfaces coated with REDV-PA/Dopa-PA nanofibers (**a**), E-PA/Dopa-PAnanofibers (**b**), and on the bare steel surface (**c**) at 2 h. (**d**) The relative adhesion of HUVECs and A7r5 smooth muscle cells on REDV-PA/Dopa-PA and E-PA/Dopa-PA coated surfaces with respect to the bare stainless steel surface at 2 h $***P < 0.0001$, *NS no significance* (Reproduced from Ceylan et al. (2011). Selective adhesion and growth of vascular endothelial cells on bioactive peptide nanofiber functionalized stainless steel surface. Biomaterials 32(34):8797–8805. Copyright © (2011) Elsevier)

HUVEC cells were seen to show enhanced proliferation on REDV-PA/Dopa-PA-coated surface compared to EPA (negatively charged PA lacking REDV sequence)/Dopa-PA-coated stainless steel surface and bare stainless steel surface as can be seen in Fig. 15.3. However, long-term retention power of morphology, viability, and proliferative capacity of HUVECs on the REDV-PA/Dopa-PA-coated stainless steel surface was observed even after 24 and 72 h by means of proper formation of filamentous actin-based stress fibers by HUVECs (Ceylan et al. 2011).

Atherosclerotic plaques can often cease the blood flow to the heart through coronary blood vessels. This severe condition can cause heart muscles to get damaged and make the heart non-functional. This condition is called heart attack or myocardial infarction. Atherosclerotic plaques, formed at the injured sites in the blood vessels, evoke inflammatory responses by profound recruitment of inflammatory monocytes from the spleen (Swirski et al. 2009; Nahrendorf et al. 2010). Hence, inhibition of this infiltration can be a therapeutic target for the treatment of myocardial infarction. Coupling of chemokine receptors, CCR-2/MCP-1, at the injured site plays a key role in this recruitment (Boring et al. 1998; Dewald et al. 2005; Serbina

and Pamer 2006). To this end, Leuschner et al. (2011) reported an approach to prohibit the infiltration of monocytes to the atherosclerotic plaques and reduce the progression of the disease. They therapeutically silenced the RNA responsible for expressing the CCR2 receptors by using siRNA against CCR2 mRNA. The authors prepared a lipid-based vesicle encapsulated with siRNA for the specific delivery of siRNA to the target sites (Love et al. 2010). Labeling of siRNAs with near-infrared fluorochromes enabled siRNAs to be traced in vivo in fluorescence molecular tomography (FMT) scanner. Flow cytometry analysis confirmed the uptake of labeled siRNAs by splenic monocytes along with dendritic cells and macrophages. PCR-assisted FACS sorted splenic monocytes from siRNA-encapsulated nanoparticle-treated mice, revealed the reduction in the number of CCR-2 expression in these monocytes. The drastic attenuation of the accumulation of these monocytes by chemoattraction of MCP-1 was shown by the migration assay of these FACS sorted inflammatory monocytes. Finally, flow cytometry analysis revealed that in myocardium of siRNA-treated mice, the infarct size was drastically reduced (Leuschner et al. 2011).

Local and systemic inflammation caused by the rupture of atherosclerotic plaques in the coronary arteries play a key pathological role in CAD. These plaques are ruptured by the macrophages that accumulate at these sites. So, the inhibition of macrophage activity is a promising therapeutic option for the treatment of CAD. Previous studies have demonstrated that statins have several effects on macrophage functioning (Mach 2004). In coronary artery diseases, statins reduce LDL uptake by macrophages and secretion of leucotactic and tissue-degrading molecules. All these effects combined reduce the number of plaque ruptures in the coronary arteries. Hence, nanotechnology-mediated delivery of statins to reduce plaque ruptures has been the focus of research. In one study, Broz et al. (2008) designed a biocompatible copolymer using poly (dimethylsiloxane) (PDMS) and poly (2-methyloxazoline) PMOXA. This copolymer was used as a vehicle for delivering hydrophilic drug, pravastatin. The copolymer was specifically targeted to macrophage scavenger receptor A1 (SRA-1) of macrophages by functionalizing the copolymer with oligonucleotide sequence PolyG. This novel formulation was successful in delivering the drug to the macrophages, as indicated by successful uptake of the polymers by the macrophages. The uptake inhibited the endocytic activity of the macrophages. The ligand-functionalized polymers were taken up only by the target cells and not by any other cell and hence no possibility of side effects. The vesicles that were taken up by the cells were seen to release the drug inside the macrophages in a time-dependent manner. This would increase efficiency at low dose (Broz et al. 2008).

Coronary occlusion can cause a major damage to the heart by interfering in the supply of oxygen and nutrients to myocardium, thus resulting in decay of cardiomyocytes. This degeneration in heart muscles can ultimately lead to heart attack or myocardial infarction (MI). In this context, promotion of new blood vessel formation by enhancing angiogenesis to restore blood supply to heart can be a therapeutic target. It is well established in our group that europium hydroxide nanorods (EHNs) are capable of promoting angiogenesis by ROS-NO-mediated signaling pathway.

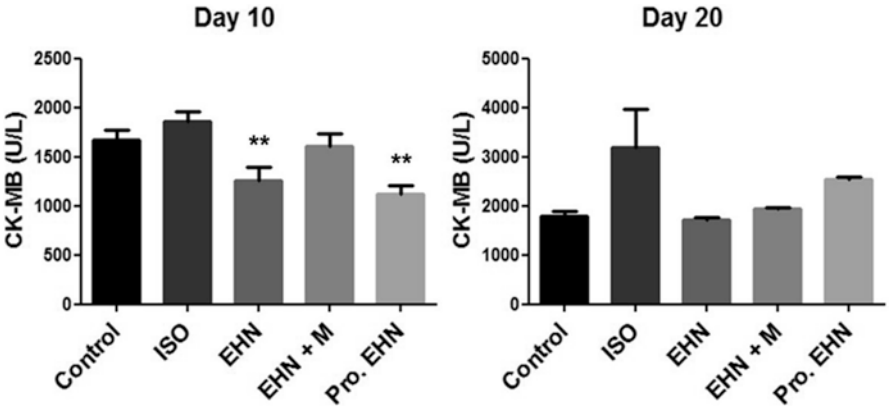

Fig. 15.4 Biochemical estimation of CK-MB (U/L) in serum isolated from blood of rat groups, collected at Day 10 and Day 20 of the study period (Reprinted (adapted) with permission from Vemuri et al. (2019). Europium hydroxide nanorods (EHNs) ameliorate isoproterenol-induced myocardial infarction: an in vitro and in vivo investigation. ACS Applied Bio Materials 2(3):1078–1087. Copyright © (2019) American Chemical Society)

The therapeutic effect of EHN on reverting myocardial infarction was validated by affirmative results of several in vitro and in vivo assays. In this study, MI was induced by treating wistar rats with isoproteronol (ISO), a well-established model for MI. In vitro assays revealed that EHN can restore viability, and reduce apoptosis of IOS-treated H9C2 cells (Vemuri et al. 2019). CPK-MB and troponin are well-known biomarkers found in serum in the case of cardiac damage. Serum biochemical analysis revealed that EHN treatment drastically reduced the levels of these biomarkers compared to untreated rat. The results are shown in Fig. 15.4. Additionally, histopathological analysis revealed normal myocardium, while ISO-treated rats suffered from myocardial degeneration. Finally, gene expression studies in heart tissues revealed that there was an upregulation of eNOS gene expression and downregulation of iNOS and TNF-α gene expression (Di Napoli et al. 2005). These results were in corroboration with the in vitro results of gene expressions with the nanorods (Nethi et al. 2015). Thus, from the study it was thoroughly proved that pro-angiogenic EHN could successfully retrieve heart functioning that was hampered as a consequence of ISO exposure (Vemuri et al. 2019).

Apart from the above-mentioned methods for curing cardiovascular diseases, one highly potential avenue for curing this chronic disease is gene therapy. Safe and efficient delivery of nucleic acids can enable upregulation (pDNA) or downregulation (siRNAs) of specific genes intrinsically associated with diseases. Although viral vectors, by virtue of their in-built ability to penetrate into the host cells and transfer genetic materials to the host nucleus, are good gene delivery vectors. This method is laden with safety concerns such as potential oncogenity, immunogenicity, and toxicity. Hence, various non-viral gene delivery strategies are being developed. Such an effort was put forward by Paul et al. (2014). They designed a biocompatible delivery vehicle with polyethylenimine (PEI) functionalized with a nanocomplex of

graphene oxide (GO) nanosheets and DNA_{VEGF}. This system was incorporated into a hydrogel composed of methacrylated gelatin (GelMA). The hydrogel nanocomposite (fGOVEGF/GelMA) enabled controlled release of the gene from the nanocomplex that was confirmed by simple agarose gel electrophoresis assay. In this assay, pre-incubated fGOVEGF/GelMA (in PBS) was run in agarose gel to detect DNA retention at various time points within 72 h. Detected bands in the agarose gel confirms slow release of pDNA from the hydrogel. Reports of ELISA using media conditioned with VEGF/pDNA hydrogel transfected H9C2 cells reveal overexpression of VEGF in those cells even after 14 days post-transfection. The author and his group used rat models to induce CAD by occlusion of the left anterior descending coronary artery. Quantitative PCR (qPCR) analysis of four inflammatory microRNAs (miRNAs) for proinflammatory role (miR-155), inflammation resolution role (miR-146a), cardiac aging and function (miR-34a), and cardioprotective role under apoptotic condition (miR-145) revealed no significant difference between the untreated and treated rats. This meant the pDNA hydrogel evoked no inflammation in the body of treated rats. Immunohistochemical staining analysis with PECAM-1 showed that there was an increase in density of capillaries in the treated group as can be seen in Fig. 15.5. This proved that the pDNA containing hydrogel induced paracrine VEGF signaling that enhanced localized vessel sprouting. Taken together, these postulates demonstrate that the newly formulated gene active GO/GelMA hydrogel can exhibit therapeutic efficacy against acute myocardial infarction without any significant side effects (Paul et al. 2014).

15.5.2 Treatment of PAD

The establishment of neovascularization as a remedy for ischemic vascular diseases has proven to be a fruitful strategy. Clinical trials have brought to the fore that various growth factors, growth factor-related genes, stem cells or endothelial precursor cells, etc., can be induced to boost up angiogenesis (Tu et al. 2015). So contemporary research on angiogenesis or curative vascular diseases focuses on the use of these pro-angiogenic moieties for promoting vascularization. But treatment approaches involving the above-mentioned elements scarcely produces satisfying clinical outcomes, as the molecules are subject to undesirable degradation, off-target effects, discrepancy in dose response, inability to trace pass cell membrane barriers, etc. (Lee et al. 2010). This drives the scientists to attempt at using nanoscale strategies as nanoparticles to overcome these barriers. Nanoparticles are acclaimed to be vehicles for controlled and sustained delivery of drugs to specific sites of the body. In the following paragraph, we will review different nanoscale approaches in effect for the treatment of PAD or PVD.

The delivery of pro-angiogenic growth factors like VEGF, FGF, HGF, PDGF, etc. (Petrak et al. 2019) via their conjugation with different nanoparticles to target sites in the body is a prevalent approach for promoting angiogenesis. Some nanomaterials in use for this purpose include biocompatible, biodegradable, polymeric

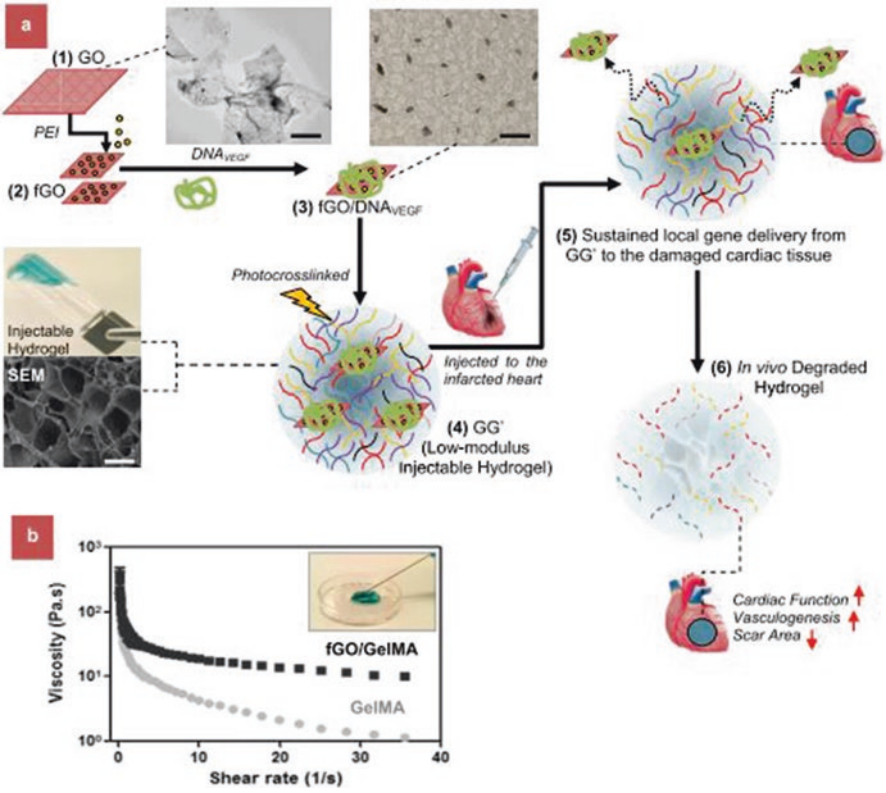

Fig. 15.5 Preparation of injectable GG′ hydrogel for AMI therapy. (**a**) Schematic of stepwise formulation process of nanobioactive hydrogel and subsequent injection to treat damaged heart with acute myocardial infarction. (1) First, GO nanosheets are functionalized by amide bond with branched PEI to form cationic fGO. (2) fGO is then surface functionalized with anionic plasmids (DNAVEGF) to form fGO/DNAVEGF as shown in TEM images. (3) These bioactive hybrids are then suspended in prepolymer of GelMA hydrogel and UV cross-linked under optimized condition to form (4) injectable fGO/DNAVEGF carrying GelMA hydrogel (GG′). (5) The latter is then intramyocardially injected in rat heart with acute intramyocardial infarction for local gene delivery of incorporated fGO/DNAVEGF nanocomplexes from GG′ hydrogel. (6) This eventually exhibits therapeutic effects by promoting myocardial vasculogenesis, which leads to reduced scar area and improved cardiac function. (**b**) Injectability of the developed GO carrying GelMA hydrogel. The viscosity of GO/GelMA nanocomposite hydrogels was monitored at different shear rates. At low shear rate, both fGO/GelMA and GelMA hydrogels had high viscosity. However, at higher shear rate, fGO/GelMA and GelMA hydrogels showed decreased viscosity. This indicates that both GelMA and fGO/GelMA were able to flow at higher shear rate and were easily injectable. The results also indicate that the addition of surface functionalized fGO to GelMA results in higher viscosity of fGO/GelMA at higher shear rate compared to GelMA. In other words, fGO reinforces the GelMA hydrogel network. Scale bar: 1 μm (Reproduced with permission from Paul et al. (2014) Injectable graphene oxide-/hydrogel-based angiogenic gene delivery system for vasculogenesis and cardiac repair. ACS Nano, 8(8):8050–8062. Copyright © (2019) American Chemical Society)

polymers like poly(lactic-co-glycolic acid) (PLGA) (Golub et al. 2010), dextran hydrogels (Hiemstra et al. 2007), alginate hydrogels (Jay and Saltzman 2009), a combination of PLGA nanospheres and alginate hydrogels (Lee et al. 2010), etc. The FDA-approved PLGA nano-biopolymer can encapsulate growth factor like VEGF. Golub et al. (2010) successfully showed that PLGA-VEGF nanoconjugation can revert ischemia, when administered in a mouse ischemic muscle. The author proved that this formulation enabled sustained release of the growth factor at the target site. In vitro bioassay like aortic ring assay showed increased sprouting. An increased number of vessels as a result of PLGA-VEGF treatment were also obtained from histopathological analysis of hind limbs and from high resolution, three-dimensional images of hind limbs observed in MicroCT (Golub et al. 2010). Some natural hormones like erythropoietin produced by kidneys and stimulating red blood cell production can also play a role in angiogenesis as observed by Li et al. (2009). The author along with his team observed that erythropoietin conjugated with gelatin hydrogel microspheres (GHM), when injected into ischemic limbs, can induce sustained release of EPO locally and stimulate angiogenesis. Non-invasive in vivo method like LDPI revealed that the formulation improved blood flow recovery in the ischemic limb. Improvements in blood vessel and arteriole density were confirmed by immunocytochemistry using markers like CD31, Ki-67, and von Willebrand factor. The authors also observed that EPO-GHM upregulated expression of Akt, p-Akt, and eNOS signaling proteins as observed in Fig. 15.6, which are directly involved in angiogenic signaling pathways. From all these observations, the author concluded that EPO-GHM formulation can play a promising role in therapeutic vascular diseases like PAD (Li et al. 2009). Polymer-based nanoparticles are also not devoid of limitations. The improper release of growth factors at target sites, undesirable coupling of the growth factors with polymer matrix, possibility of alteration of growth factors in terms of morphology and function as a result of effect of temperature, pH values during polymer formation, etc., are some of the constraints of using biopolymer-based nanoparticles (Zhang et al. 2009). Inorganic nanoparticles, by virtue of their high surface to volume ratio, have advantages over nanopolymers and are also more suitable as carriers in the blood stream (Zhang et al. 2006). Zhang et al. (2006) used FDA-approved inorganic mesoporous silica nanoparticles (MSN) as a drug delivery agent for bFGF drug. The nanoformulation was found to be biocompatible and promote cell proliferation of HUVEC cells. Simultaneously, the porous structure of the nanoparticle can allow absorption of cell staining dye. This indicates that MSNs can be an efficient drug delivery system and an excellent biomaterial applicable in bio-imaging (Zhang et al. 2009). Another very promising approach for treating ischemic diseases is the use of stem cell therapies. But this avenue is also ridden with a lot of hurdles like poor cell survivability, poor retention of cell morphology during long-term ex vivo culture, incompetence in the production of a sufficient number of stem cells for transplantation, etc. Recent advances in science and technology have moved far ahead by overcoming these limitations and introducing cell-free approaches by the use of exogenous stem cell mobilizers. These species can assemble stem cells at target sites and induce desired effects. Park et al. (2018) reported the use of an effective

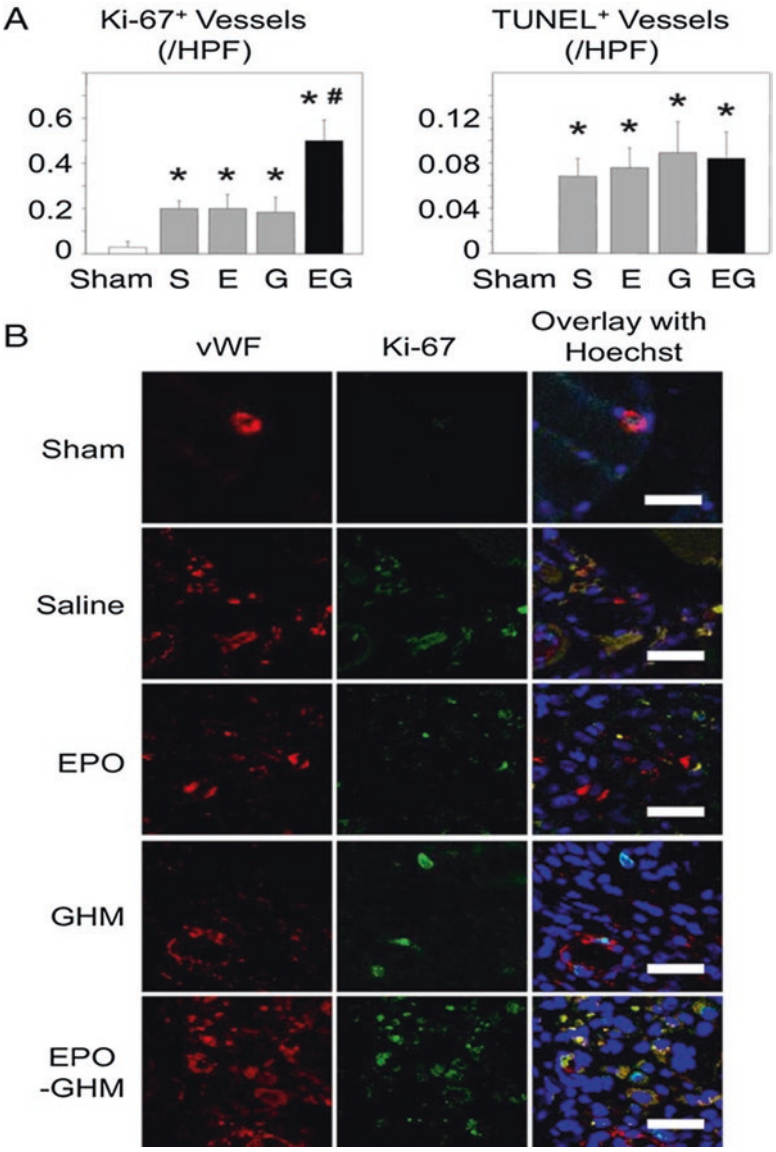

Fig. 15.6 Effects on the proliferation or apoptosis of vessels in ischemic limbs. (**a**) Graphs showing the proliferating or apoptotic index of vessels at 8 weeks after ligation. $*P$ 0.05 versus the sham group; $^{\#}P$ 0.05 versus the other control groups. (**b**) Confocal micrographs of the ischemic limb specimen taken from the mice showing the double immunofluorescent labeling of von Willebrand factor (red fluorescence) with Ki-67 (green fluorescence). Scale bars: 20 m (Reproduced with permission from Li et al. (2009). Sustained release of erythropoietin using biodegradable gelatin hydrogel microspheres persistently improves lower leg ischemia. Journal of the American College of Cardiology 53(25):2378–2388.Copyright © (2009) American College of Cardiology)

MSC and EPC mobilizer, substance P peptide, conjugated with a high-density lipoprotein (HDL)-mimicking nanodisks for the treatment ischemia in diabetic mice. The nanoconjugation was found to impart stability and increase circulation time of substance P. The peptide, on the other hand, improved blood perfusion by initiating revascularization and blood vessel formation, as was observed by LDPI. Immunocytochemistry analysis by CD31 and SMA staining as depicted in Fig. 15.7 revealed that arteriolar and capillary density was increased by HDL-SP treatment in comparison to the untreated group. In addition to these, SP-HDL was also observed to exhibit the suppression of pro-inflammatory cytokine secretion, macrophage infiltration, and enhancement of anti-inflammatory cytokine secretion along with M2 macrophage polarization at the ischemic site (Park et al. 2018).

Gene therapy is a significant approach for manipulating the functions of cells. It allows for prolonged production of proteins in target tissues. Hence, the application of gene therapy for the purpose of therapy in many diseases is a common practice. In this regard, treatment for PAD is no exception. Various gene carriers have been

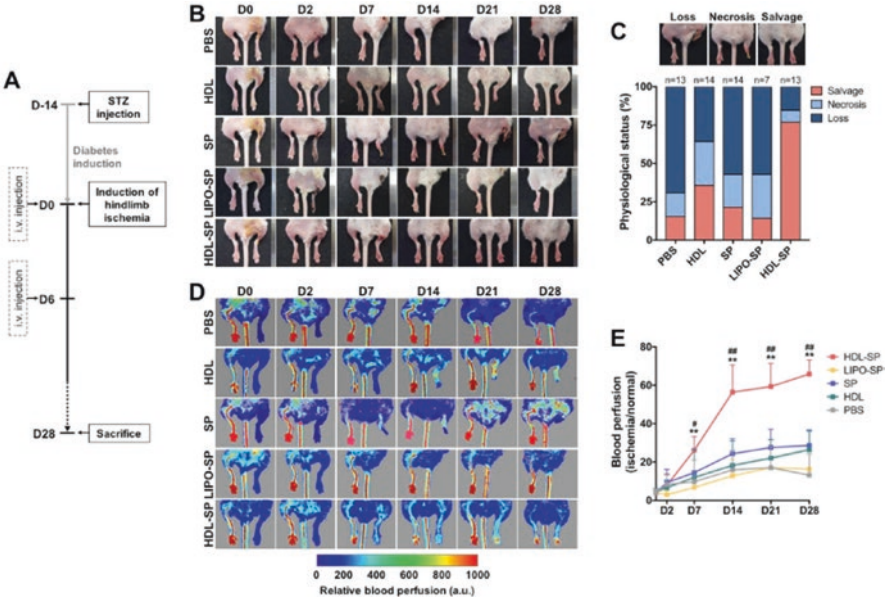

Fig. 15.7 Improved ischemic limb salvage by HDL-SP administration in a diabetic hindlimb ischemia model. (**a**) Experimental scheme showing the time-line of diabetes and ischemia induction, injection treatments, and sample analyses. (**b**) Representative images of diabetic ischemic limbs of each group. (**c**) Scoring of physiological status of diabetic ischemic limb 28 days after ischemic injury and injection treatments (n = 7–14). (**d**) Serial analyses of blood reperfusion in ischemic limb using Doppler Flowmeter. (**e**) Quantification of blood reperfusion by calculating the perfusion ratio of ischemic limb to normal limb on days 0, 2, 7, 14, 21, and 28 after ischemic injury and injection treatments (n = 6; $^{**}P < 0.01$ versus PBS, HDL, and LIPO-SP groups, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus SP group) (Reproduced from Park et al. (2018). High-density lipoprotein-mimicking nanodisks carrying peptide for enhanced therapeutic angiogenesis in diabetic hindlimb ischemia. Biomaterials 161:69–80. Copyright © (2018) Elsevier)

used such as viral vectors, CRISPER/CAS9 system, lipids, polymers, non-viral vectors like direct injection of plasmid DNA, electroporation of naked, colloidal DNA, gene gun, electrospray, etc. These approaches also have several drawbacks like transfection efficiency, potential toxicity, inconsistent stability, off-target shots, etc. (Kim et al. 2017). The advancement of nanotechnology offers a very promising avenue to overcome these shortcomings. Nanoparticles can operate as vehicles for carrying specific genes/RNAs, etc., to the cell. These nanocarriers can protect cells from degradation, cross cell membrane barriers, escape endosome, and also translocate cargo inside the nucleus. For the treatment of PAD, scientists have used a wide variety of gene delivery nanovehicles, including polymeric materials like polyamidoamine dendrimers (PAMAM), polyethylenimine (PEI), heparin/chitosan/PLGA nanoparticles, peptides, inorganic materials like gold, silica, carbon nanotubes, etc. These carriers are employed to carry pro-angiogenic genes like VEGF, bFGF, CD151, and other growth factor genes (Liu et al. 2017). A combination of nanopolymers, polyethylenimine (PEI) and 2,6-pyridinedicarboxaldehyde (PDA), both of which are biodegradable in vivo, can form a copolymer named PDAPEI to deliver pDNA of growth factor VEGF. It is well known that pDNA has high transfection efficiency and this makes it an attractive target element for gene therapy. Liu et al. (2017), in his study, reported the development of potential cure for limb ischemia. The author's novel formulation of bio-copolymer can successfully deliver VEGF pDNA to ischemic site in limb and bring about remedial effects. From immunehistological studies using CD31 and BrdU, the author contemplated that there was improvement in proliferation and microvessel density. In vitro cytotoxicity study in HUVEC proved the compound to be biocompatible. Additionally, the author and his team also found out that there was an increased expression of VEGF-A protein in ischemic hind limb muscles after PDAPEI/pDNApolyplex treatment (Liu et al. 2017). FDA-approved PLGA biopolymer was reported to have even better transfection efficiency. Kang et al. (2008) made a comparative study on PEI/VEGF-pDNA and PLGA/VEGF-pDNA to evaluate their efficacy in PAD treatment. The author observed that PLGA nanosphere/pDNA can be a more efficient carrier for targeted VEGF gene delivery than PEI/VEGF pDNA. This observation was supported by the fact that ischemic limbs treated with PLGA/VEGF had a higher concentration of VEGF than that with PEI/pDNA. Prevalent in vitro bioassay like immunocytochemistry using CD31 and BrdU antibodies determined increased endothelial cell proliferation and increased microvessel density in PLGA/pDNA compared to PEI/pDNA and control group (naked pDNA). Histological analysis showed enhanced neovascularization in PLGA/VEGF-treated limbs rather than in PEI/VEGF-treated limbs. Apoptosis level of ischemic tissues treated with PEI and PLGA revealed that there was more apoptotic tissue in PEI/VEGF-treated muscles than in PLGA/VEGF-treated muscles. All these evidences and cytotoxicity study proved that PLGA/VEGF pDNA is biocompatible and a better inducer of angiogenesis due to high transfection efficiency (Kang et al. 2008).

Liposomes form an excellent drug delivery system as they are biodegradable, biocompatible, less toxic, have prolonged duration of action, and can deliver both

hydrophobic and hydrophilic molecules to the target site with high specificity. Due to their similarity to cell membranes, they are taken up easily by cells, when they are conjugated with specific cell surface antigens. Hence, liposomal delivery of drugs, important growth factors, genes, and even RNA inhibitors to the target sites is a very dependable strategy for composing curative medicines. Like other well-explored domains in this regard, ischemia is also an area of research for translating liposome-oriented therapeutic devices into clinical practices (Tu et al. 2015). The basic strategy of liposome-mediated treatment is the delivery of pro-angiogenic peptides to target ischemic sites via liposomes. Such an approach was conducted by Hwang et al. (2016), who evaluated the efficacy of the treatment of limb ischemia, using liposomes loaded with pro-angiogenic peptides. The liposomal formulations were administered in mice induced with limb ischemia, by both intra-arterial and intramuscular routes. In order to advance the circulation of liposomes in the blood without eliciting an immune response, the investigators coated the liposomes with PEG groups. The authors observed that both the routes of administration were equally effective in improving limb perfusion and promoting angiogenesis. Simultaneously, in vitro studies also showed that the liposomal formulation promoted migration and tube formation abilities in endothelial cells. Taken together, the authors proved that liposomal formulations offer a reliable avenue for the treatment of PAD (Hwang et al. 2016). The eventuation of angiogenesis necessitates an intrinsic interaction and activation of growth factors, inflammatory cytokines, components of signaling pathways, and proliferation of cells like endothelial cells. So extraneous induction of growth factors or cell signaling components can influence the angiogenesis process. Hence, scientists are hypothesizing that delivery of growth factors, or cell signaling components like receptors, signaling molecules can be useful in the treatment of vascular-related diseases. In this context, study by a group of researchers revealed that liposomal delivery of FGF-2 growth factor and its co-receptor syndecan-4 can elicit angiogenesis, as observed by enhanced tube formation, migration, and pro-angiogenic signaling response in endothelial cells. In vivo studies revealed that these syndecan-4 proteo-liposomes possess neovascularization potential in ischemic tissues as can be seen in Fig. 15.8. Thus scientists showed that the model of delivering a growth factor along with its receptors or co-receptors can enhance therapeutic applications amenable to vascular-related diseases like PAD (Jang et al. 2012).

The therapy with bone marrow-derived stem and progenitor cells is also a highly potential option for treatment of chronic diseases. Several research groups around the world are constantly working for formulating an effective therapeutic treatment option for angiogenesis-related diseases. Deveza et al. (2016) made an effort to formulate a stem cell–based therapy to treat PAD. The author and his group formulated poly(β-amino ester) (PBAE)-based nanoparticles and complexed them with CXCR4 gene and transfected them in ADSCS ex vivo depicted in Fig. 15.9 (Deveza et al. 2016).

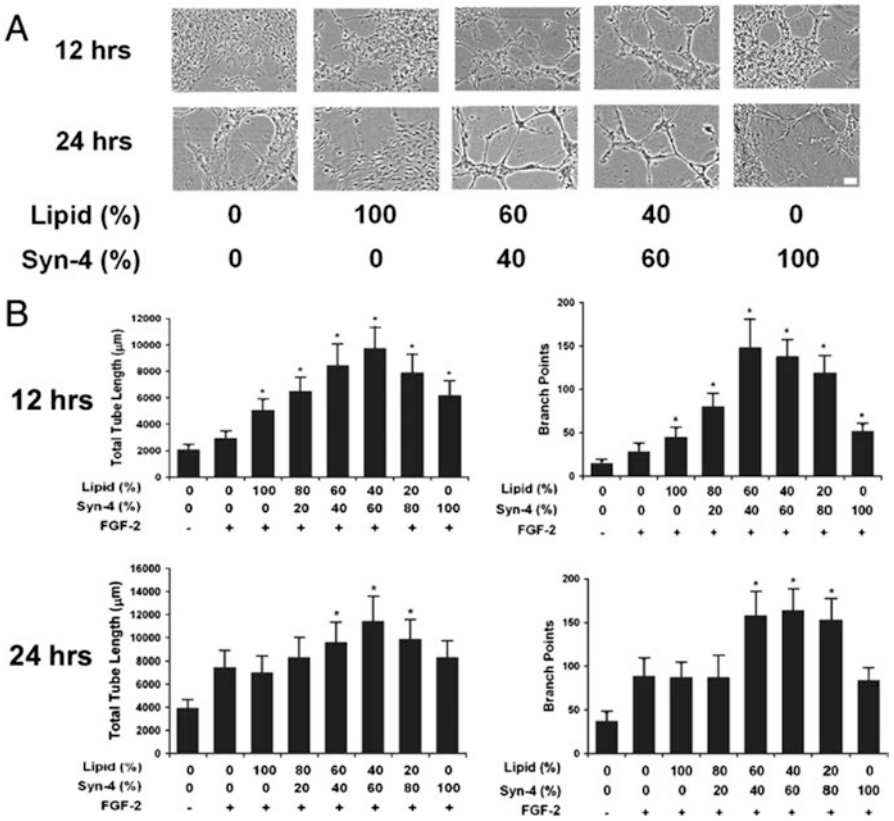

Fig. 15.8 Syndecan-4 proteoliposomes enhance in vitro tube formation in combination with FGF-2. HUVECs were seeded on extracellular matrix and exposed to various treatments. In vitro tube formation was quantified by measuring total tube length and the number of branchipoints. All samples were treated with FGF-2 (10 ng/mL) unless labeled otherwise. (**a**) Phase-contrast micrographs of endothelial cells in Matrigel after 12 h of treatment (scale bar, 20 μm). (**b**) Quantitative analysis of tube length formed by endothelial network after 12 h of treatment. The number of branch points in endothelial networks after 12 h. The length of tubes in the endothelial network following 24 h of incubation. Branch points in endothelial networks after 24 h of treatment. ∗Statistically different from FGF-2 group ($P < 0.05$) (Reproduced from Jang et al. (2012). Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle. Proc Natl Acad Sci USA 109(5):1679–1684. Copyright © (2012) United States National Academy of Sciences)

Fig. 15.9 (continued) planted cells measured 24 h (**f**) and 7 days (**g**) after cell transplantation. (**h**) Quantitative BLI signal of transplanted GFP(+)/Luc(+) ADSCs demonstrates prolonged post-ischemia induction (expressed as percent of BLI signal relative to day 0 signal). *C* CXCR4, *CV* CXCR4/VEGF, *V* VEGF, *G* GFP, *rel.* relative, *h* human. All data are reported as mean ± standard error, $n = 8$. $*P < 0.05$ compared with GFP-ADSC controls cell survival by CXCR4-overexpressing groups over 14 days (Reproduced with permission from Deveza et al. (2016). Polymer-DNA nanoparticle-induced CXCR4 overexpression improves stem cell engraftment and tissue regeneration in a mouse hindlimb ischemia model. Theranostics 6(8):1176. Copyright © (2016) Ivyspring International Publisher)

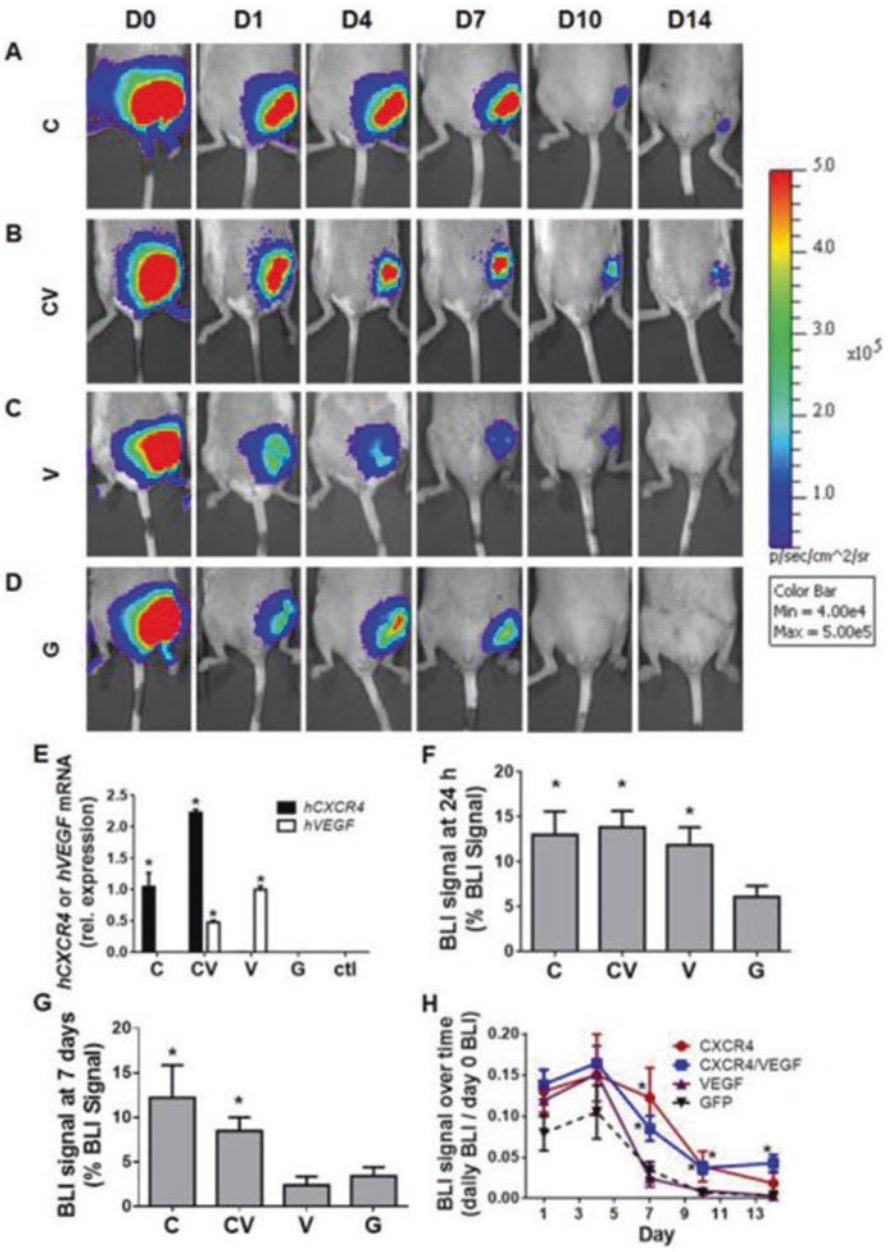

Fig. 15.9 CXCR4-overexpressing ADSCs displayed improved cell engraftment and survival in murine models of hindlimb ischemia. (**a**–**d**) Representative BLI of mouse ischemic hindlimbs transplanted with GFP(+)/Luc(+) mouse ADSCs transfected with CXCR4 (**a**), CXCR4/VEGF (**b**), VEGF (**c**), or GFP (**d**) over 14 days post-ischemia induction. (**e**) Confirmation of human CXCR4 and human VEGF overexpression in transplanted ADSCs by qRT-PCR performed on harvested tissue at 4 days post-procedure (data are expressed as mRNA expression relative to single transfection Fig 15.9 (continued) with hCXCR4 or hVEGF). Percentage of BLI signal remaining from trans

15.5.3 Treatment for Cerebral Ischemic Disease

Currently many treatments are available in the market for the therapy of CID, which includes various neuroprotective drugs, antiplatelet therapy, and clot busting drugs all of which fall under the category of chemotherapeutic agents. But there have been major drawbacks while using these drugs because of the intervention of the transport system and also by the BBB that prevents the drug molecules to cross. Hence there is an utmost need of the nanoparticles as they bind with both hydrophilic and hydrophobic types of drugs so that the drugs become stable and can easily cross the BBB as well as the transport system (Kaviarasi et al. 2019). Also from various reports, it can be seen that more than one type of compound can be incorporated to the nanoparticles (Hu et al. 2012). Also there have been reports that nanoparticles' surface can be modified to attach a large number of targeted ligands for the site-directed action along with the incorporated drugs to the diseased site in order for proper treatment (Kaviarasi et al. 2019). There have been various types of nanoparticles used for the therapeutic application of ischemic stroke; among them are polymeric nanoparticles and liposomal carriers.

Sarkar et al. (2017), in a very innovative experiment, incorporated melatonin hormone into the PLGA nanoparticles in order to exploit the antioxidant property of the hormone in the cerebral stroke region. The treatment was given to cerebral ischemic female rats with reperfusion injury. After the treatment, the normal form of anti-oxidant enzymes is restored and matrix metalloproteinase-9 also helped in the suppression of lipid peroxidation along with decreased dysfunction of the mitochondria (Sarkar et al. 2017). In another experiment, Reddy and Labhasetwar (2009) encapsulated the antioxidant enzyme super oxide dismutase (SOD) with poly(D,L-lactidecoglycolide) nanoparticles for scavenging the area of cerebral ischemia of free radicals. The experiment was performed in the rat model with cerebral ischemia having reperfusion injury. The injection of the nanoparticles was given during the time of reperfusion through the intracarotid artery in order to prevent edema formation and maintain the structure of the BBB. The outcome of the experiment was that there was more survivability of the animals treated with nanoparticle-conjugated enzyme as compared to the free enzyme-treated animal, and it also reduced the diseased area (Reddy and Labhasetwar 2009).

Other than the antioxidant route, the method of gene silencing is also reported to prevent the expression of the genes that induce a cycle of events that cause the death of the neurons by the help of small interfering RNA (siRNA). Since there is a problem for the siRNA to cross the BBB, Wang et al. (2018) used PEGylatedpoly(L-lactide) nanoparticles to encapsulate siRNA, which have been complexed with a derivative form of cholesterol to target C3, which is a complement factor. Small interfering RNA (siRNA) inhibits the activity of this component, which acts as a mediator in the pathway of neuroactivation and also helps in the activation of the microglia. When the siRNA-conjugated nanoparticles were injected into the animal, the siRNA could easily move through the BBB and silenced the C3 component (Wang et al. 2018). As discussed earlier, most of the drugs face the problem of

crossing the blood brain barrier while entering the ischemic site. So scientists have started to exploit the function of leukocytes to reach the ischemic site of the brain by using the process of neuroinflammation. It acts as a best time to export the drugs to the diseased site. In this context, Zhang et al. (2017a) used dendrigraft poly-L-lysine (DGL) nanoparticles conjugated with PGP, which is reported to have affinity toward neutrophils. As a result of this, the neutrophils are attracted to the diseased site; also the action of the catalase enzyme was protected from getting destruction and the cells received the drugs efficiently as shown in Fig. 15.10. Because of the increased catalase activity at the diseased site, the area of the ischemic disease was significantly reduced (Zhang et al. 2017a). Table 15.2 depicts the overall use of nanoparticles in the treatment of ischemic diseases.

15.6 Ischemic Reperfusion Injury (IRI)

The ischemic reperfusion injury is one of the key characteristics of many different ischemic diseases, including peripheral artery disease, cerebral artery disease, and myocardial infarction (Cryer 1997). The reperfusion injury is marked by the blockage of the blood vessels due to which there is insufficiency in the blood flow. This leads to shortage of oxygen and nutrients, disruption in the process of oxidative phosphorylation, and reduction in the production of adenosine triphosphate (ATP). This lack of oxygen supply results in reperfusion, and cells are recovered from the oxygen insufficiency. But the backdrop of this process is upregulation in the ROS production along with lipid peroxidation, opening of the aperture of the mitochondrial permeability pore(mtp), damage of the DNA, and cytokine release leading to inflammation (Daiber et al. 2017b). But ROS is essential in IRI and the normal ischemic disease because it is directly involved in the increase in the smooth muscle cells and also has a role in angiogenesis through vascular endothelial growth factor (VEGF) and also hypoxia-inducible factor (HIF-1) (Maulik 2002). Along with this, peroxynitrite is also involved in IRI and ischemia that is chronic in condition. During the phase of IRI, uncoupling of eNOS occurs because of hypoxia and oxidative stress due to which there is upregulation in the ROS production. This causes cell destruction and disruption in the vascular functioning by an increased level of peroxynitrite and superoxide (Daiber et al. 2017a). The increased peroxynitrite also causes upregulation in platelet action and constriction of the microvasculature (Daiber et al. 2017c).

In the second phase of ischemia that is in reperfusion injury, more amount of oxygen leads to upregulation of the oxidative stress and decreased NO, leading to different processes such as lipid peroxidation, formation of cytokines, and matrix metalloproteinase activation. All these lead to cell damage due to an increase in inflammation and toxicity (Rathore et al. 2008). Many recent studies highlight the fact that regulation by means of redox reaction, which involves thiol oxidation and networks of glutaredoxin, reduces ischemia as well as IRI (Watanabe et al. 2016). The reperfusion injury occurring in the case of cerebral ischemia involves

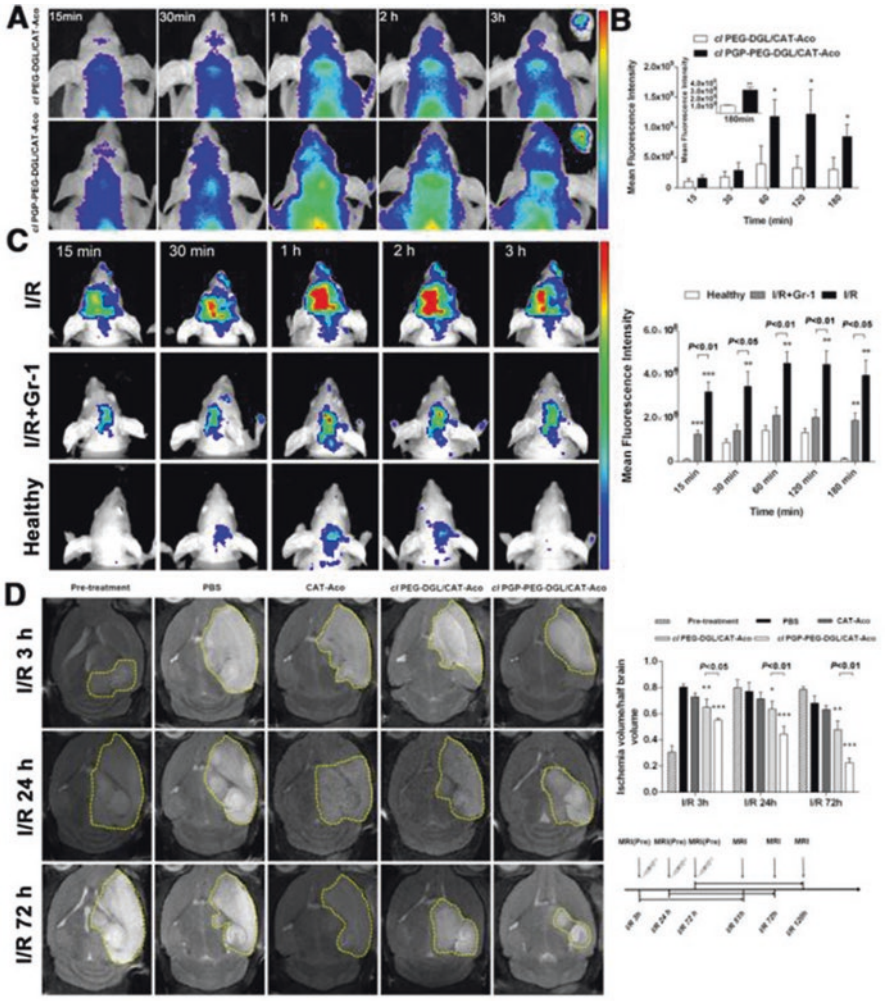

Fig. 15.10 (**a**) In vivo fluorescence images of the brains of nude mice subjected to MCAO after the administration of cldendrimer/CAT-Aco NPs labeled with CAT-BODIPY; ex vivo NIR fluorescence image of harvested brain 3 h post-treatment. (**b**) Semi-quantitative ROI analysis of the average fluorescence intensity from the BODIPY 650/665-labeled NPs in ischemic brain (mean ± SD, $n = 3$), $*P < 0.05$, $**P < 0.01$ vs. cl PEG-DGL/CAT-Aco. (**c**) In vivo fluorescence images of nude mice brain after cl PGP-PEG-DGL/CAT-Aco NPs administration with or without the injection of anti-Gr-1 antibody (mean ± SD, $n = 3$). NPs were labeled with CAT-BODIPY. $**P < 0.01$, $***P < 0.001$ vs. the healthy. (**d**) In vivo T2W-MRI images of ischemic mouse brain at 48 h post-injection of PBS, CAT-Aco alone (250 U/mouse), cl PEG-DGL/CAT-Aco, and cl PGP-PEG-DGL/CAT-Aco (mean ± SD, $n = 6$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the PBS group (Reproduced with permission from Zhang et al. (2017a) Direct macromolecular drug delivery to cerebral ischemia area using neutrophil-mediated nanoparticles. Theranostics 7:3260–3275. Copyright © (2017) Ivyspring International Publisher)

Table 15.2 Nanoparticles for treatment of ischemic diseases

Nanoparticles	Diagnostic approach	Reference
PLGA nanoparticles	VEGF conjugated and helps in mouse ischemic muscle disease	Golub et al. (2010)
PLGA nanoparticles	Melatonin hormone and helps in cerebral stroke region	Sarkar et al. (2017)
Gelatin hydrogel microspheres	Erythropoietin conjugated and helps in ischemic limbs	Li et al. (2009)
High-density lipoprotein (HDL)-mimicking nanodiscs	MSC and EPC mobilizer, substance P peptide and helps in ischemia in diabetic mice	Park et al. (2018)
Polyethylenimine (PEI) and 2,6-pyridinedicarboxaldehyde (PDA)	MSC and EPC mobilizer, substance P peptide and helps in ischemia in diabetic mice	Liu et al. (2017)
Liposome	FGF-2 growth factor and co-receptor syndecan-4 conjugated and helps in vascular-related diseases like PAD	Jang et al. (2012)
Poly(D,L-lactide coglycolide) nanoparticles	Antioxidant enzyme super oxide dismutase (SOD) and helps in cerebral ischemia	Reddy and Labhasetwar (2009)
PEGylated poly(L-lactide) nanoparticles	siRNA and C3, a complement factor conjugated and helps in cerebral ischemia	Wang et al. (2018)
Europium hydroxide nanorods (EHNs)	Myocardial infarction	Vemuri et al. (2019)
Gelatin hydrogel microspheres (GHM)	Erythropoietin conjugated and treatment of PAD	Li et al. (2009)

neuroinflammation where the immune cells, which infiltrate produce many inflammatory substances to employ different immune and glia cells. This results in the recovery of the tissue from the injury and maintains its integrity (Xanthos and Sandkuhler 2014). But when a high amount of activation of microglia occurs, it results in an increase in inflammation by the release of pro-inflammatory cytokines such as TNF-α, different types of interleukins such as (IL)-1β, IL-12, and IL-6, and also interferon γ(INF-γ) (Hernandez-Ontiveros et al. 2013). This is mainly caused by the leakage in the BBB (Chodobski et al. 2011). Also, the pro-inflammatory cytokines have certain effects: they increase the amount of free radicals along with ROS, cyclo-oxygenase-2, and other neurotoxic molecules. This results in the death of the neuronal cells (Hernandez-Ontiveros et al. 2013; Kabadi and Faden 2014). Since neuroinflammation takes place in the non-microbial environment, the host receptors are activated by the damage-associated patterns, which in turn are relieved from the cytoplasm in response to cell death or injury (Eltzschig and Eckle 2011). This activates innate responses because of a large amount of pro-inflammatory substance generation and causes ROS-dependent lipid peroxidation, damage of the DNA, and ultimately cell death (Chen and Nunez 2010; Garry et al. 2015).

15.7 Future Perspectives and Challenges

The emergence of nanoparticles has been a boon to the world of medical and diagnostic applications. With its unique features and properties, nanoparticles can be used as a perfect alternative for the commercially available treatments and diagnostic tools that have many disadvantages and problems. Therefore, nanoparticles are used for the theranostic applications of various diseases, including ischemia, which has become one of the leading diseases affecting a majority of the world population. There are still no proper methods of diagnosis and treatment for the ischemic patients that have been affected. In the case of different types of ischemia, there have been a large amount of medicines and drugs being introduced to repair the damage and to decrease the infarcted area, but in the translational system, they were not so successful. Research journals, scientific experiments, FDA approval, and also clinical trials of nanoparticles for theranostic applications are increasing with each day (Anselmo and Mitragotri 2016).

To increase the efficacy of nanoparticles, a few aspects that should be included are discussed in the following section. The first one of it is designing of nanoparticles that are able to target new receptors and ligands and can show dual targeting ability. The second is the usage of lipid-based liposomes, dendrimers, polymers that exhibit sensitivity to different pH, temperature, etc., to enhance the delivery facility (Frias et al. 2004). Protein-based nanoparticles should be increased in order to increase the facility of using them as vectors (Bejarano et al. 2018). Also metal nanoparticles exhibit fluorescence activity, which can be exploited for the diagnostic purposes of ischemic diseases (Azharuddin et al. 2019). The addition of growth factors, drugs, and various targeting ligands to nanoparticles enhances the treatment procedure (Liu et al. 2017). Nanoparticles are of small size so they can easily enter cell organelles, helps in gene editing, and can cross semipermeable barriers (De Jong and Borm 2008). For example, researchers made nanoparticles that help in the growth of new blood vessels from the pre-existing ones, known as angiogenesis, and are using this new mode of therapy for the treatment of ischemic diseases (Vemuri et al. 2019).

But there still exist challenges, faced by the patients for the treatment of ischemic diseases. One of them includes the administrative route of drug delivery system; for example, in the case of cerebral ischemia, it has been observed that those drugs that are administered through the intravenous or the intranasal route is not so effective in treating brain ischemia than those that are administered through intra-arterial route. Hence, nanoparticles, drugs, and other therapeutics need to be made in competence with the delivery route (Masserini 2013; Dong 2018). Another challenge that needs to be addressed is the usage of least toxic and more effective nanoparticles as a treatment agent. There can be direct toxicity of nanoparticles since it depends upon the structural composition of the nanoparticle (Ambesh and Angeli 2015). Also the production cost, manufacturing issues, and the stability of nanoparticles pose a challenge for their usage and entry in the market of clinical trials (Fernandes et al. 2018). The other challenges of using nanoparticles are that the developmental unit should be extremely clean in order to avoid any sort of contamination because it can

hamper the utility of the therapeutic application of nanoparticles. The retention of nanoparticles inside the body of the animal is also a major concern since it can possess long-term effects and therefore major changes need to be done for the easy clearance of the nanoparticles (Zhang and Monteiro-Riviere 2009). Also regarding the toxicity of nanoparticles, pharmacokinetics and pharmacodynamics studies should be performed in order to avoid toxicity issues (Choi and Han 2018).

15.8 Conclusion

In summary, nanoparticles are regarded as the new-age materials for bringing a revolution in the field of diagnostics and treatment. The use of nanoparticles has helped in the diagnosis and treatment of ischemic diseases, which remain a major challenge in the medical field. In the context of treating ischemic diseases, several pre-clinical trials have demonstrated the benefits of using nanoscale strategies for safe and efficacious methods of diagnosis and treatment. In this chapter, we have laid emphasis on the mechanisms involved in the pathology of ischemic diseases and how nanotechnology can target those intricate mechanisms in order to ameliorate the irregularities associated with the diseases. Nanoparticles themselves act as therapeutic agents along with other conjugated products and have helped to decrease the infracted diseased area. Besides, nanotechnology-based gene therapy, stem cell therapy, growth factor-based therapy, etc., have also made a significant impact on the treatment of ischemic diseases. In addition to treatment methodologies, this chapter has also thrown light on the different diagnostic procedures that nanotechnology has. Researchers have attached radioactive compounds to nanoparticles so that when reaching the targeted site, they can be easily detected. Although the existing challenges need to be addressed yet, the future prospects of nanoparticles are indispensable. Briefly, we can conclude that nanoparticles are the new emerging technology in the theranostics field.

Acknowledgments Financial support from Nano Mission-DST (SR/NM/NS-1252/2013; GAP 570), New Delhi, to C.R.P. is duly acknowledged. P.B.T. and S.H. are thankful to CSIR, New Delhi for supporting Junior Research Fellowship. The authors are thankful to the Director, CSIR-IICT for his support, encouragement, and keen interest in this work. IICT Manuscript No. IICT/Pubs./2019/288 dated August 5, 2019 for this manuscript is duly acknowledged.

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Chapter 16
Nanoantibiotics: The Next-Generation Antimicrobials

Ramu Manjula and Manjunath Chavadi

Abstract From last three decades, more studies are being done to discover new antibiotics to combat multidrug-resistant (MDR) pathogens. Nanoparticles with antimicrobial activity were found to a new replacement for the conventional antibiotics. These nanoantibiotics are having their own intrinsic antimicrobial activity to kill microbes in the pathogenic cell. They are also called nanocarriers as they are used to deliver the drugs at action site. There are various metal NPs and ceramic NPs which can be used in combination with conventional antibiotics to target multiple mechanisms to kill microbes. Though there are many advantages in applying them in different medical purposes, it is associated with many challenges such as toxicity and large-scale production. The present chapter discusses about the present scenarios and applications of nanoantibiotics.

Keywords Antimicrobials · Multidrug-resistant (MDR) pathogens · Nanoparticles · Nanoantibiotics

16.1 Nanoparticles and Nanotechnology

"Small is beautiful and powerful" (Khanna 2008) is the hallmark feature of nanoparticles (NPs). Then, the question comes what these NPs are what is so fascinating about it that the world is behind it. Well, the primary concept of NPs was presented by Richard Feynman in a lecture entitled "There's plenty of room at the bottom" at the American Institute of Technology in 1959. The prefix nano is derived from the

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_16

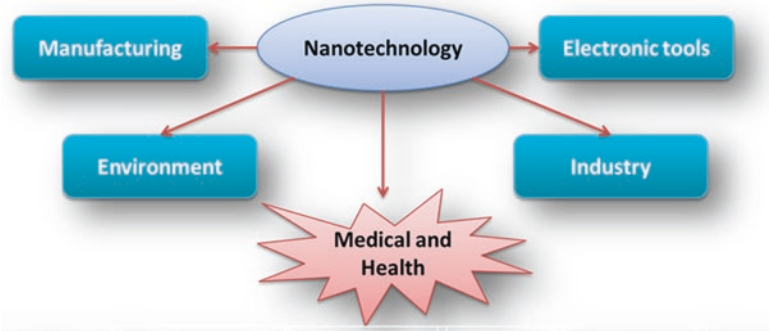

Fig. 16.1 Application of nanotechnology in different fields

Greek word Nanos meaning "dwarf" that refers to things of one billionth [109 m] in size (Narayanan and Sakthivel 2010). The most fascinating thing about NPs is they bridge the gap between bulk materials and atomic or molecular structures, which attracted scientific interest all around the globe.

Nanotechnology plays a role in many areas of the science and technology. They are used in different sectors of industries, in the production of electronic tools, in different methods of environmental protection, and also in medical and health care (Fig. 16.1). To get sustainable energy, at the same time to reduce the burden of toxicity, a solar panel with nanotechnology involved is becoming an efficient replacement for conventional solar cells. Nanotechnologies are also used in the production of batteries with a higher power density that holds electrical charge longer (Jalaja et al. 2016). Nanotechnology can also be applied in the identification of impurities during water purification and soil remediation. In agri-food productions at various stages, nanotechnology is utilized for application of pesticides, protection of crops from diseases, and genetic manipulation of plants and finally in the postharvest management (Sekhon 2014; Prasad et al. 2014, 2017a, b, c; Bhattacharyya et al. 2016).

In medical field, it has vast application in gene and drug delivery (Suri et al. 2007), diagnostic and imaging tools (Koo et al. 2005), tissue regeneration (Gu et al. 2013; Walmsley et al. 2015), biodegradation, and antimicrobial applications (Harakeh et al. 2017). Gold NPs are already being used in the treatment of cancers and other diseases (Jain et al. 2012). Nitrogen-doped carbon nanotubes are also used in the treatment of cancers (Vardharajula et al. 2012). The novel gene sequencing technologies are using advanced solid-state nanopore materials for the novel gene sequencing that enabled single-molecule detection at low cost and high speed with minimal sample preparation and instrumentation (Liu et al. 2016).

16.2 Nanoantibiotics

The application of antibiotics is significantly important in infection-prone conditions during safe surgical procedures, organ transplants, and chemotherapy. However, in recent years, a rapid emergence of resistance to almost all class of antibiotic has emerged as a real threat to the society. After the evaluation of multidrug-resistant bacteria, many diseases have become untreatable, and from past decade, almost 22 different antibiotics are developed to overcome the threat (Butler et al. 2017). The antimicrobials kill bacteria by binding to some vital compounds of bacterial metabolism or disturbing the normal cellular activities. The β-lactams like penicillin and cephalosporin inhibit bacterial cell wall synthesis; tetracyclines, clindamycin, macrolides, metronidazole, and quinolones inhibit protein synthesis; sulfonamides and trimethoprim have an inhibitory effect on enzyme synthesis. Some bacteria are untreatable by the antibiotics such as vancomycin-resistant *S. aureus* (VRSA) and methicillin-resistant *S. aureus* (MRSA). Since their spores are highly resistant and very thick, it is difficult to enter the drug inside the cell and not affected by antibiotics. This shows that treating these virulent bacteria with antibiotics is extremely difficult.

In recent decades, antibiotics formulated in polymeric NPs have demonstrated enhanced antimicrobial activities and anti-MRSA activities, compared with nonpolymerized forms of penicillin and N-methylthio β-lactams (Turos et al. 2007a, b). The NPs that show antimicrobial activity are effective replacements for the present-day antibiotics and are called "nanoantibiotics." To increase the efficacy of conventional antibiotics, multiple antimicrobial agents are incorporated in the NPs for concurrent delivery (Zhang et al. 2010). Nano-antimicrobials are roughly divided into two categories: (a) NPs that have a direct killing effect on pathogens and (b) NPs that act as a carrier and increase the antimicrobial activity of the encapsulated drug (Jamil et al. 2017).

There are multiple advantages of using nanoantibiotics against conventional antibiotics. Some of them are as follows (Huh and Kwon 2011):

- Nanoantibiotics tackle with multiple biological pathways found in broad species.
- Preparation or synthesis is cost-effective.
- They can prolong shelf-life.
- They can withstand harsh conditions, such as high-temperature sterilization, under which conventional antibiotics can get inactivated.
- Nanomaterials offer controllable targeted delivery for the antibiotics with improved solubility, reduced side effects, and increased cellular internalization.

16.2.1 Mechanism of Action of Nanoantibiotics

The nanomaterials with the high surface area-to-volume ratios and unique chemicophysical properties contribute to the effective antimicrobial activities (Weir et al. 2008). The mechanism of action is not understood; however, the disruption of the

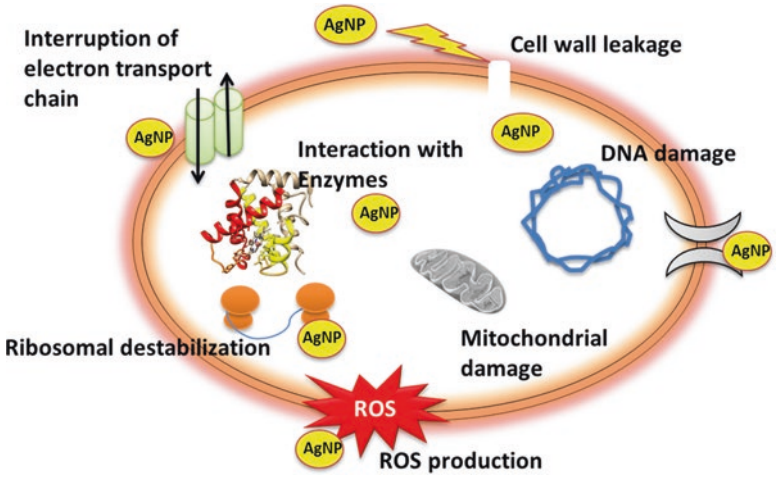

Fig. 16.2 Antimicrobial mechanism of nanoantibiotics

bacterial cell was observed (Fig. 16.2). The factors influencing the activity of nanoantibiotics are size and shape, concentration, chemical composition, photoinactivation, and target species. The integrity of the membrane is disrupted by the attachment of NPs (Thill et al. 2006; Aziz et al. 2014, 2015, 2016, 2019). Potential antimicrobial mechanisms are as follows:

- Cell wall disruption and leakage of cell components
- Production of reactive oxygen species (ROS) that damages the viral and cellular components
- Interruption of energy transduction and mitochondrial damage
- Inhibition of enzyme activity
- DNA damage and inhibition of DNA synthesis
- Disruption of protein synthesis by ribosomal destabilization
- Interruption of electron transport chain

In 2009, a study also demonstrated that metal NPs failed to show the antimicrobial resistance of naturally occurring bacteria (Muhling et al. 2009).

Various metal and metal oxide NPs, such as copper oxide (CuO), titanium dioxide (TiO_2), silver oxide (Ag_2O), silicon (Si), zinc oxide (ZnO), gold (Au), magnesium oxide (MgO), and calcium oxide (CaO), are well demonstrated as efficient antibiotics (Table 16.1). All these NPs possess various applications in food, medical, and industrial areas (Edson and Kwon 2016). Silver NPs (Ag NPs) are the most studied material among all nanomaterials and are made from biological materials to get "green"-synthesized Ag NPs. The second most studied is the gold NPs (Au NPs) which actually does not show antimicrobial activity on its own (Chatterjee et al. 2011). In combination with conventional antibiotic, Au shows effective inhibition against MDR

Table 16.1 Nanoparticles with antimicrobial properties

Nanoantibiotics	Mechanism of action	Applications
Ag NPs	Disruption of the cell membrane and electron transport; DNA damage	Dressing for surgical wound and diabetic foot; coatings for medical devices; portable water filters; antibacterial and antifungal agent
ZnO NPs	Cell membrane damage; H_2O_2 production	Antibacterial creams; lotions and ointment; surface coating of the medical device; mouthwash
TiO_2 NPs	Production of ROS; cell membrane and wall damage	Antibacterial agent; food sterilizing agent; air purifiers; water treatment systems
Au NPs	Interaction with cell membranes; strong electrostatic attraction	Photothermal therapy; adjuvant treatment, antibacterial and antifungal agent
Mg NPs	Inhibition of microbial enzymes, damaging cell membranes ROS induction, biofilm inhibition	Effective against *E. coli* and *Bacillus megaterium* and moderate activity against spores of *Bacillus subtilis*
Silica NPs	Disrupt microbial biofilms, Lipid peroxidation of cell membrane	Treatment against MDRs
Chitosan	Increased permeability and rupture of the membrane; chelation of trace metals; enzyme inactivation	Drinking water disinfectants; bacteria immobilizer; microbiocide in biomedical products
Fullerenes	Destruction of cell membrane integrity; enhancing activity of infiltrating neutrophil	Potential disinfection applications
CNTs	Cell membrane damage by ROS; oxidation of cell membrane proteins and lipids	Antibacterial agent; biofouling-resistant membranes; water filter; surface-coating
NO-releasing NPs	NO release and production of ROS	Infected wound and diabetic foot treatment
Nanoemulsion	Membrane disruption; disruption of the spore coat	Antimicrobial inhaler; anti-biofilm agent; nasal application; vaccine delivery agents
Gelatin	Destabilization of membrane	Food additive and immunoassay

Ag NPs silver nanoparticles, *ZnO NPs* zinc oxide nanoparticles, *TiO_2NPs* titanium oxide nanoparticles, *Au NPs* gold nanoparticles, *CNT* carbon nanotubes, *NO* nitric oxide

microbes. Ceramic NPs such as titanium dioxide (TiO_2) acts as a photocatalytic antimicrobial agent (Allaker and Memarzadeh 2014). Silica NPs are mainly used for the drug encapsulation in pharmaceutical companies. Out of all, ZnO has better stability during the production, and hence, it is commonly used for medical applications.

16.2.2 Stimuli-Responsive Linkers

For most microbial infections, the action and expression of virulent factors depend upon the conditions such as osmolarity, carbon dioxide, pH, temperature, oxygen, amino acids, and iron levels, and hence, they are responsible for efficient

multiplication in the host. The pathogens may use these factors as signals to detect their environment (Mekalanos 1992). These factors can be used as stimuli-responsive linkers to get the synergetic effect of nanoparticles with antibiotics. The pH-responsive linkers can withstand the high acidic environment as in stomach and the high basic environment as in the gastrointestinal tract. The microbes such as *Helicobacter pylori*, *Agrobacterium tumefaciens*, or *Vibrio cholera* grow in an acidic environment of the body. Polymers with carboxylic, sulfonic acid, or amino groups such as poly(L-histidine), poly(methacrylic acid) (PMAA), and poly(acrylic acid) (PAA) have been commonly used for their pH-responsive functionalities (Edson and Kwon 2016). Change in pH aids the release of drugs from the linkers.

The microbes like *Salmonella typhimurium*, *Bordetella pertussis*, *Escherichia coli*, *Vibrio cholera*, and *Shigella* sp. rely on temperature-dependent environmental signals. Change in temperature induces the disruption of intra- and intermolecular electrostatic and hydrophobic interactions which results in collapse or expansion. Materials become soluble in the upper critical solution temperature (UCST) and insoluble in lower critical solution temperature (LCST) (Roy et al. 2013). LCST materials including N-isopropylacrylamide (NIPAM), N-vinylcaprolactam (NVCl), methylvinylether (MVE), and UCST materials are based on a combination of acrylamide (AAm) and acrylic acid (AAc). The response of light is also an excellent stimulus for the antimicrobial activity. Near IR laser treatment is less harmful and penetrates deep into the tissue. These materials possess light sensitive functionalities like azobenzene, spiropyran, or nitrobenzyl groups. TiO_2 as photocatalyst can be used against *E. coli* (Liou and Chang 2012). The presence of redox-response functionalities in nanomaterials is useful for reduction of disulfide bonds.

Some microorganisms create enzymes as virulent factors to thrive in microenvironments. In enzyme-responsive systems, enzymes are used to destroy the polymer or its assemblies which can release the drugs. A linker with pyroglutamyl-peptidase I, a protease of *S. aureus* can selectively allow the release of antibody at the infection site (Rozema et al. 2015).

The combination of drug molecules with nanoantibiotics exhibits excellent antimicrobial properties and can be engineered to have effective therapy against MDR pathogens. One of the examples is combination of chitosan and sulfamethoxazole against *Pseudomonas aeruginosa* (Tin et al. 2009). According to study, silver NPs have shown to significantly increase antibacterial activities for drugs such as kanamycin, ampicillin, erythromycin, and chloramphenicol when tested against *E. coli*, *S. typhi*, *S. aureus*, and *M. luteus.* In this kind of therapy, two different mechanisms of microbe can be targeted simultaneously by nanoantibiotics and conventional antibiotics.

16.3 Antimicrobial Nanomaterials

The different types of antibacterial NPs are metals and metal oxides, naturally occurring antibacterial substances, carbon-based nanomaterials, and surfactant-based nanoemulsions. The metal nanoparticles have shown promising results compared to other polymers as they possess high surface area-to-volume ratio.

16.3.1 Silver as Antimicrobial Agent

Silver metal has been an antimicrobial agent from ancient time, and it is used in the form of silver, silver nitrate, and silver sulfadiazine. After the discovery of new antibiotics, their usage has been reduced. Although colloidal silver products are available as health supplements, it is illegal in the USA. Ag NPs attack the respiratory chain, and cell division that kills the cell, by releasing silver ions, induces the bactericidal activity (Klasen 2000). Synergistic antimicrobial effects were seen against Gram-positive and Gram-negative bacteria when the Au NPs are combined with penicillin G, amoxicillin, erythromycin, and vancomycin. It exerts only minimal adverse effects on health when using metallic silver, and higher dosage may affect the mitochondrial activity (Braydich-Stolle et al. 2005). Though the Ag NPs have been increasingly applied in the biomedical or pharmacological fields, relatively little research has been done in clinical medicine.

16.3.2 Gold Nanoparticles

Au NPs are well-known antibacterial agents, and they have also been used in detection of bacteria. They exert electrostatic attractions to the negatively charged cell membrane. Gold nanoshells, nanocages, and nanorods are used to treat bacterial infections where laser pulses are used for irradiation (Johnston et al. 2010). The combined effect of antibiotics and Au NPs (Au NPs coated with streptomycin, gentamicin, and neomycin) has been found to have high activity (Pissuwan et al. 2010). A laser-induced hyperthermia effect coupled with bubble formation around clustered Au NPs leads to selective killing of *S. aureus* (Muhling et al. 2009). In summary, Au NPs are promising adjuvants for replacing antibiotic therapy in treating serious MDR bacterial infections.

16.3.3 Aluminum Copper Nanoparticles

According to a recent study, meso-Ag/Al_2O_3NPs demonstrated inhibitory effect on *P. aeruginosa* and *S. aureus* (Buckley et al. 2008). At high concentration, Al_2O_3 NPs inhibit the microbial growth. Though the copper ions are part of many enzymes, free ionic Cu^{2+} at a high concentration can generate toxic effects by generating ROS that disrupts the amino acid synthesis (Esteban-Tejeda et al. 2009). Environmental Protection Agency (EPA) reported the antimicrobial activity of almost 300 different copper surfaces in 2008 after which metallic copper was studied as a major antimicrobial agent. The copper NPs are found to have more affinity toward *Bacillus subtilis* and hence act as superior antibiotics compared to Ag NPs. Copper oxide (CuO) is cheaper than silver, easily miscible with polymers, and relatively stable chemically and physically.

16.3.4 Zinc Oxide Nanoparticles

Against some foodborne bacterial infections, ZnO NPs can be used as antibiotics. Meantime, they are also helpful in managing the agricultural food products. Since ZnO NPs are with low production cost, UV blocking ability, and white appearance, they are more advantageous than Ag NPs. The ZnO NPs act on cell membrane to disrupt the lipid and proteins and generate H_2O_2 and Zn^{+2} which are toxic to bacteria (Sawai 2003; Bhuyan et al. 2015).

16.3.5 Titanium Dioxide Nanoparticles

TiO_2 is the commonly used photocatalyst and used in photocatalytic antimicrobial activity where it disrupts cell membrane. Near-UV or UVA irradiation induces the antimicrobial activity of TiO_2 NPs, and its activity depends on the size, intensity, and wavelength of light source. They are active against *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecium*, and *C. albicans.* Treatment of TiO_2 NPs also produces the ROS such as hydroxyl and peroxides which would kill the microbes. A combination of Ag and TiO_2 NPs showed strong light independent antimicrobial activities, and this combination was tested to have strong antibacterial activity against both *E. coli* and *B. subtilis* spores (Hamal et al. 2010). As they are having activity against *Lactobacillus acidophilus*, they could also be used in dental surgeries and problems.

16.3.6 Antimicrobial Peptides and Chitosan

Chitosan is a partially deacetylated chitin that exhibits antimicrobial effects against bacteria, viruses, and fungi. However, they are more effective against fungal and viral infections than bacterial one. Low molecular weight chitosan is more active against the cell wall of Gram-positive bacteria compared to high molecular weight (Fernandes et al. 2010). In combination with sulfamethoxazole, chitosan possesses synergetic antimicrobial activity against *P. aeruginosa*. Chitosan causes agglutination at the cell wall and increases the cell permeability which finally leads to intracellular leakage. It also inhibits many enzyme activities by chelating metals from enzyme. The oleoyl-chitosan NPs with different internalization pathway can kill both *E. coli* and *S. aureus* (Xing et al. 2009). Usage of chitosan has several advantages and disadvantages. They are cost-effective with high antimicrobial activity and low toxicity for humans.

16.3.7 Nitric Oxide (NO)-Releasing NPs

NO is a free radical involved in immune responses. NO is mostly used in combination therapy and found to be a promising NP against MRSA (Hetrick et al. 2008). Since NO is a free radical, NP-based scaffolds can store large NO loads that can be released under aqueous conditions at physiological pH and temperature. The NO-releasing silica NPs are well developed to kill bacteria (Gram positive and Gram negative) and fungi (Hetrick et al. 2008).

16.3.8 Carbon Nanotubes (CNTs)

Carbon tubes are tube-like structures made of only carbons with covalent interactions. There are single-walled nanotube (SWNT) and multi-walled carbon nanotubes (MWCNTs) with the diameter of 1–5. SWNTs are used for water purification, inactivation of *E. coli*, and poliovirus and removal of MS2 phages (Fang 2004). Hence, they can be used in the preparation of water filtration membranes to avoid biofilm formation. They have also been used in photothermal therapy by delivering CNT nanoclusters to an infected area and selective destruction of drug-resistance microorganisms upon near-infrared irradiation (Maisch 2009).

16.3.9 Fullerenes (C60) and Fullerene Derivatives

Fullerenes are aqueous insoluble NPs that are used as antimicrobial agents. Some studies demonstrated that antibacterial activity of nC_{60} to prokaryotic cells is mediated through the lipid peroxidation in cell membrane (Fang et al. 2007), and few studies found that they act via ROS-independent mechanism (Lyon et al. 2008). The antimicrobial activity of carboxyfullerene is mediated by insertion into the cell wall, followed by disruption of the cell membrane structure. In comparison with vancomycin, alkylated C60-bis(N,N-dimethylpyrrolidinium iodide) derivatives affect the respiratory chain and inhibit bacteria growth. Fullerol (polyhydroxylated fullerenes $[C_{60}(OH)_n]$) shows lower toxicity compared to nC_{60} and can also be used as a drug carrier that bypasses the blood ocular barriers.

16.4 Advantages of Nanoantibiotics

Nanotechnology is the key to a current world in the fields of food and agriculture, construction materials, medicine, and mechanical and electrical engineering. Some of the advantages of nanoantibiotics are as follows:

- It is cost-effective.
- It improves the bioavailability of drugs by enhancing the solubility.
- It protects the drug from premature degradation, and hence, minimum dose can be used to treat the infection.
- It reduces the dose-dependent toxicity of the drug by reducing its dose of intake.
- Targeted drug delivery releases the drug at the site of action; hence, only infected area gets the drug delivered.
- There is sustained and controlled release of drugs.

16.5 Challenges for Nanoantibiotics

The benefits of nanotechnology are widely studied in different fields including medicine, but the potential effects or side effects of these nanosized particles are not well clarified. The main challenges in biological applications are toxicity and large-scale synthesis. Therapeutic administration of NPs may generate multiorgan nanotoxicity. The prolonged exposure of metal and carbon nanoparticles generates toxicity. For instance, silver NPs provide more benefits as nanoantibiotics; however, longer exposure is also toxic to the cells. It causes permanent pigmentation in skin and eyes. Similarly, fullerenes and nanotubes also exert toxicity at certain concentration and solvents. Administration of NPs through i.v. has a possibility to be accumulated in bone marrow and the spleen, liver, and lung, and also inhaled NPs can reach the brain, heart, lung, liver, and spleen due to its smaller size and efficient uptake by cells (De Jong and Borm 2008). There are no standard methods to assess the dose of NPs to administer at different organs or tissues.

Another challenge associated with nanoantibiotics is the large-scale production. There are chemical (bottom-up) and mechanical (top-down) pathways to generate them (Edson and Kwon 2016). The conventional chemical and physical methods are often toxic and nonbiocompatible. Generally, the chemical methods are used to synthesize NPs at low cost for high volume production; however, their drawbacks include contamination from precursor chemicals and the use of toxic solvents which generate hazardous by-products (Thakkar et al. 2010). Another method used to generate nanoparticles is an emulsion system which is a bottom-up approach. The method is limited to certain NPs that are soluble in organic solvents.

In nanoscience and nanotechnology, green synthesis of NPs is the most attentive area which involves the economic and eco-friendly approaches. In contrast, biological methods were regarded as possible eco-friendly and alternative methods for the synthesis of NPs (Prasad 2014; Prasad et al. 2016, 2018). So, much importance is given to synthesis of NPs by this route. However, the entities that concentrate on the industrial production of nanoantibiotics should consider the pros and cons of the methodology they consider and the toxic effects associated with the by-products.

16.6 Conclusion

To combat the MDR pathogens such as *S. aureus*, it is important to have new antibiotics and nanoantibiotics that serve as a better replacement for the conventional antibiotics. Because of high surface area-to-volume ratio and unique physicochemical properties, nanoantibiotics are promising therapy for the diseases. Nanoparticles can be used in different medical applications such as NP-based dressings, NP-coated medical devices, nanogels, and nanolotions. Of the many different approaches to overcome antimicrobial resistance, using NPs as antibiotic carriers seem to hold the highest promise. Though rapid progress is seen, there are still some topics of concern with respect to toxicity, especially to those that will manufacture the materials. More attention is needed in the field of large-scale production of nanoantibiotics as well.

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Chapter 17
Nanoprobiotics: When Technology Meets Gut Health

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Abstract Nanotechnology is a fast-rising industry not defined by a single field of research, but as the convergence of disciplines, such as chemistry, biology, physics, mathematics, and engineering, which exploits the benefits of nanoscale dimensions and characteristics for application in the macroworld. Current applications vary widely from nanorobotic industry to simple household items. However, the combination of such phenomena with probiotic science, another emerging and potentially promising area for the prevention and treatment of several human gastrointestinal and extraintestinal disorders using beneficial microorganisms, gives birth to "nanoprobiotics," a field that focuses on the application of nanoscience into the probiotic-related world. In this chapter, we will navigate through the basic nanotech and probiotic knowledge and the current technologies employed with success for probiotic delivery and, ultimately, discuss what possibilities lie ahead in the nanoprobiotic future.

Keywords Encapsulation · Gut · Health · Nanotechnology · Prebiotics · Probiotics · Synbiotics

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17.1 Nanotechnology: The Who, What, Why, How, and Where

17.1.1 Who

The first definition describing technological processes that allowed the achievement of higher precision and ultra-small dimensions (on the nanometer scale, 10^{-9} m) was attributed by Norio Taniguchi of Tokyo Science University, where he stated that "Nano-technology mainly consists of the processing of separation, consolidation, and deformation of materials by one atom or one molecule" (Taniguchi 1974). Born from the Greek word "nanos," meaning "dwarf," the prefix nano- refers to the factor of 10^{-9}, in the international system for units of weights and measures. Interestingly, the concept of the engineer operating at a molecular level is not novel. In the year 1959, during a lecture titled "There's plenty of room at the bottom" given at the American Physical Society meeting at Caltech, Nobel Laureate Physicist Richard Feynman suggested that laws of physics allowed the controlled arrangement at an atomic level, envisioning the technology for material manipulation and control at a nanoscale, without even naming it as such (Feynman 1960).

17.1.2 What

Following decades of breakthrough in this ever-modernizing era, despite the many definitions that can be encountered (Theis et al. 2006), the consensus lies on the criteria that nanotechnology is about the design, characterization, production, and application of materials and systems by controlling the shape and size at the nanoscale, more specifically below 100 nm (Bhushan 2010). It is, however, important to differentiate it from nanoscience which is, in essence, an extension of the existing science areas to the study of the phenomena and manipulation of living and non-living matter into the nanometer (Hornyak et al. 2009; Jeevanandam et al. 2018). In fact, another criterion that should be included in the definition is that a nanoparticle (NP) or nanomaterial must be engineered, or synthetically produced (Bhushan 2010). It is important to note that NPs are not a human invention, and the deliberately manufactured NPs are in fact a minority. They exist widely in nature in the form of photochemical and volcanic activity products, mineral composites (such as oxides and carbonates), and magnetotactic bacteria (Griffin et al. 2017; Jeevanandam et al. 2018). Additionally, incidental NPs have been created as the byproducts of processes such as combustion of diesel fuels (Sioutas et al. 2005). Regarding the engineered NPs, they can be further classified based on the number of dimensions that fall outside the nanometer range, meaning that a zero-dimensional nanostructure would have all its dimensions fitted into the nanoscale, whereas a two-dimensional (2D) nanostructure would have one dimension within the nanometric diameter and two in the macrometric diameter (Table 17.1).

17.1.3 Why

The nanoscience application phenomenon is an effort that requires the involvement of various research fields and different areas of technology and lies on the premise that fabrication of materials, devices, and systems at this scale will allow the enhancement of the design and production of materials with subsequent applications in traditional industries (Abdullaeva 2017a). The improvement in the bulk properties of materials is based on the following rationale: nanostructures affect the mechanical, chemical, and electrical properties when employed, by increasing the main material surface area. Secondly, when the matter is at the nanoscale, its behavior is ruled by quantum effects, which affect the electrical, optical, and magnetic performance of materials (Davies 2007).

17.1.4 How

Irrespective of the type of materials utilized and the purpose of the fabrication, there are two main techniques to manipulate matter into the nanoscale: top-down and bottom-up approaches (Fig. 17.1). In the top-down approach, the bulk material undergoes a restructuration producing smaller components, the nanomaterials (e.g., lithography) (Liddle and Gallatin 2016). This usually requires laborious processes, such as milling, that entail more costs, are energy expensive, and involve the usage of toxic reagents, and sometimes the resulting product is not reproducible. In contrast, in bottom-up approaches, nanomaterials are assembled from their basic building blocks (atoms, molecules), which are usually nano-objects with the appropriate features for the bulk material. This can further occur via "self-assembly," in which there is a natural structural rearrangement determined by the interactions among the

Table 17.1 Nanostructures of dimensional classification

Nanoscale dimensions	Examples
0D "zero-dimensional"	Quantum dots
1D "one-dimensional"	Nanofibers, nanotubes, nanorods, and nanowires
2D "two-dimensional"	Nano-coatings, nanofilms, nanolayers, and graphene
3D "three-dimensional"	Bulk powders, dispersions of nanoparticles, bundles of nanowires, and nanotubes as well as multi-nanolayers

Adapted from Ngô and Van der Voorde 2014

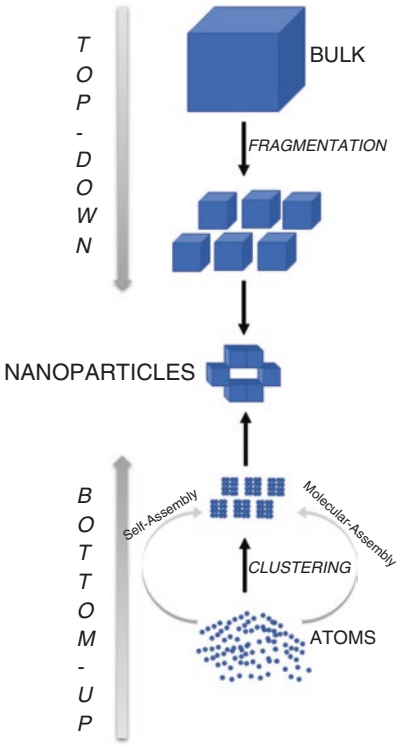

Fig. 17.1 "Top-down" and "bottom-up" approaches for the synthesis of nanomaterials (Adapted from Rawat 2015; Galstyan et al. 2018)

individual components, requiring little intervention as possible (e.g., protein folding, chemical synthesis) (Whitesides and Grzybowski 2002) and via "positional assembly" (or molecular assembly), in which atoms and molecules are purposefully manipulated. Overall, positional assembly offers greater control over fabrication, but is still viewed more as a visionary concept, not yet suitable for industrial applications (e.g., robotic molecular manufacturing systems) (Trobe and Burke 2018). Bottom-up "self-assembly" occurrences are abundant in nature (e.g., protein folding, lipid bilayer formation, colloid crystallization, bacterial colonies formation, weather patterns, solar systems) (Whitesides and Grzybowski 2002), and chemists have been exploiting for centuries the self-organizational physicochemical principles for the design of molecular structures with specific properties (Wong et al. 2009). Some of the most important bottom-up processes in nanomaterial production that lead to the manufacturing of complex structures with controlled dimensions and morphology are, among others, precipitation reactions (microemulsions, micelles, and liposomes) (Silva et al. 2015) and sol-gel processes (production of a gel from powder-shaped materials) (Gonçalves 2018).

17.1.5 Where

For all the reasons previously highlighted, it should be clear by now that "nanotech" products have found their way into several areas of industry. Indeed, the technology has already become industrially relevant (e.g., chemical and automobile sector), such as in the form of nanocomposites, nanoclays, nanocoatings, and nanopaints, among many others (Stark et al. 2015). Granted, nanomaterials are not exclusively used for industrial purposes or even "sci-fi"-type scenarios, but also incorporated into many commercially available products with the purpose of exploiting the benefits of the nanoscale at lower costs (Iavicoli et al. 2014). The application of nanotechnology into common products such as UV sunscreen filters, containing titanium dioxide and zinc oxide (Dastjerdi and Montazer 2010); textiles, containing nanosilver particles with antimicrobial properties (Morais et al. 2016); and microelectronics (nanochips, nanobatteries, touch screens, sensors) (Abdullaeva 2017b) is relatively well-known and becoming ubiquitous in our daily lives.

Notwithstanding the great impact that facilitates our daily lives, the extremely diverse facets of nanotechnology research and development also revolutionized biological technologies, which benefited, among others, the food and biomedical sectors.

17.2 Nanotechnology in Human Health

In 1996, 40 years after Feynman's famous speech, experimental chemist Richard Smalley received a Nobel Prize for the discovery of fullerene, a C60 spherical molecule. He recognized the significance of nanosized materials in medicine and related areas stating that "human health has always been determined on the nanometer scale; this is where the structure and properties of the machines of life work in every one of the cells in every living being. The practical impact of nanoscience on human health will be huge" (Burgess 2012).

Indeed, nanobiotechnology cross-links the concepts of nanotechnology into biological systems, enabling the control over biological processes that occur at the nanoscale (including the microbial physiology). This emergent field benefits areas intimately connected to human health, such as food-related products and medicine, by improving and developing new analytical tools, diagnostic techniques, and therapeutic protocols (Boulaiz et al. 2011). In nanomedicine, the employment of "nanotech" knowledge and tools for the diagnostics, treatment, and prevention of diseases and traumatic injuries, two distinct but interconnected areas have been positively impacted: diagnostics/prevention and therapeutics (Freitas 1999). Nanoparticle-enabled diagnostics is an increasingly emerging area, with NPs being used as

contrast media for imaging technologies such as magnetic resonance and computed tomography, enhancing both the state-of-the-art sensitivity and accuracy, translating into high-resolution cellular imaging (Li et al. 2016), which has a major impact specially in early detection diseases like cancer (Kumagai et al. 2013) and Alzheimer's (Keating 2005). In terms of nanomedicine therapeutics, regenerative medicine (e.g., tissue engineering) is still in early development, with only existing concepts and prototypes (Xavier et al. 2015; Krueger et al. 2016). In fact, most nano-applications are focused on drug delivery vehicles, seeking improved substance-targeting, controlled release and bioavailability (Alvarez et al. 2017). These include (1) polymeric micelles, (2) dendrimers, (3) polymeric NPs, (4) polyplexes, and (5) liposomes, all possessing different chemical structures and biological characteristics (Blanco et al. 2009).

Nanotechnology impact in food science and food microbiology is evident in operations such as agricultural productivity enhancement (Sekhon 2014; Prasad et al. 2014, 2017a, b, c) and water treatment which can benefit the quality of available foods and drinking water (Singh et al. 2017; Prasad and Thirugnanasanbandham 2019). Furthermore, the multidisciplinary facets of nanotechnology are also expected to significantly enhance functional foods and nutraceutical development. The improved delivery of bioactive compounds and micronutrients (better encapsulation agents generate optimized protection, targeting, and integration in food matrices), increased bioavailability thereof, and food contaminant detection (e.g., rapid and sensitive isolation and detection of foodborne pathogens such as *Escherichia coli* or *Staphylococcus aureus*; and of chemicals) are some of the practical applications of nanotechnology that rapidly translate into better human health (Singh et al. 2017). Alongside, with the more recent emergence of probiotics as an exciting and promising strategy to prevent and treat inflammatory/metabolic dysbiosis-related conditions (Almeida et al. 2019; Markowiak and Slizewska 2017), this "miniaturization" can add value to a wide spectrum of immobilization techniques such as encapsulation. As a result, "nanoprobiotics" arises as a term standing for the integration of probiotic organisms in nanotechnology. In fact, the nanoprobiotics concept might overlap with nanomedicine, since it relates to the production of targeted delivery and release of specific probiotics and bioactive food ingredients (Sekhon 2010). Nanomaterials for encapsulation comprehend traditional agents, such as alginate-Na, implemented in nanoemulsions, liposomes, and micelles (Silva et al. 2015) which can be integrated into food matrices (functional foods) or delivered as therapeutic formulations. Thus, inspired by nanotechnology, both food and pharmaceutical industries have achieved great advances in the development of novel delivery systems into a probiotic-related field. As discussed later, this has contributed to enhancement of the effectiveness and efficiency of these bioactives to improve human health.

17.3 Probiotics

17.3.1 Concepts, Health Benefits, and Requirements

The word "probiotic" comes from the Greek word meaning "for life," and it is a term widely used mainly in nutrition and health contexts (Markowiak and Slizewska 2017). Historically, the concept of probiotics was first put onto a scientific basis by the work of Elie Metchnikoff performed at Pasteur Institute. In fact, Metchnikoff hypothesized that the intake of fermented dairy products with lactic acid bacteria, such as yogurt, was linked with enhanced health and longevity in the elderly Bulgarian population (Metchnikoff 1907). Thereafter, the definition of probiotics has been modified and evolved over time. The current definition of probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" is widely accepted, and it was initially generated in 2001 by Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) (2001) and recently maintained and reinforced with a minor grammatical correction by International Scientific Association for Probiotics and Prebiotics (Hill et al. 2014).

In the last decades, the use of probiotics as a way to prevent and treat a panoply of human gastrointestinal and extraintestinal disorders has gained a growing number of supporters, among clinicians and researchers (Figueroa-González et al. 2011; Pintado et al. 2014). Indeed, the consumption of probiotics has been linked with several health benefits including improvement of intestinal health and alleviation of symptoms associated with intestinal inflammatory conditions (Saez-Lara et al. 2015), enhancement of lactose tolerance (Oak and Jha 2019), enhancement of the immune response, and prevention of allergic disease (Wang et al. 2019), hypocholesterolemic effect (Ishimwe et al. 2015), and anticancer effects (Marinelli et al. 2017). As depicted in Fig. 17.2, several mechanisms of action have been associated with probiotic benefits, including production of antimicrobial substances like hydrogen peroxide, bacteriocins, or organic acids (Vandenbergh 1993), competition with pathogens for adhesion sites (Collado et al. 2007) and nutrients (Deriu et al. 2013), degradation of toxins and blocking of toxin receptors (Castagliuolo et al. 1996), as well as modulation of immune responses (Ashraf and Shah 2014).

Currently, the microbial strains must meet certain requirements to be considered as a potential probiotic. According to the guidelines suggested by the FAO/WHO, every potential probiotic strain must be correctly identified, followed by several in vitro assays with the aim to investigate their functional properties (FAO/WHO 2001). Resistance to gastrointestinal conditions, adherence to mucus and/or intestinal epithelial cells, and antimicrobial activity against potential pathogens are the main properties that potential probiotic must possess. There are numerous commercial probiotics in the market, but still, there is a demand for novel probiotic strains with better properties than existing ones. Thus, additional probiotic characteristics should be considered including cholesterol reduction ability, antioxidant, and anticancer effects (Shokryazdan et al. 2017). To note that, probiotic characteristics are not related with the genus or species of a microorganism, but with certain strains of

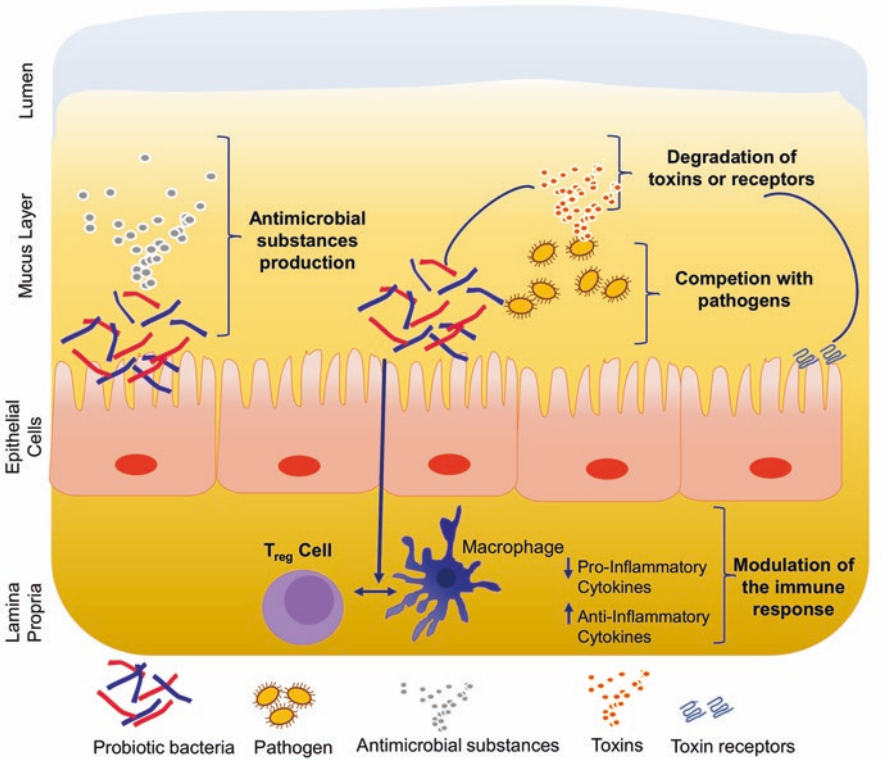

Fig. 17.2 Mechanisms of action of probiotics (Adapted from Almeida et al. 2019)

a particular species (Jacobsen et al. 1999). After taxonomic identification and functionality properties research, potential probiotics must be characterized in terms of safety parameters and technological usefulness (FAO/WHO 2001). The safety parameters are related to the origin of strain, the absence of association with pathogens, and the antibiotic resistance profile. Meanwhile, technological robustness of probiotic strains is related to their ability to survive and maintain their biological properties throughout the storage and distribution processes (Markowiak and Slizewska 2017).

17.3.2 Conventional and Potential Next-Generation Probiotics

Conventionally, probiotics have been isolated from such biological sources as the gut or derived from fermented foods (such as yogurts, fermented milk, and cheeses). Importantly, they have been classified as generally recognized as safe at the strain level by the Food and Drug Administration or included in qualified presumption of

safety at the species level by the European Food Safety Authority (EFSA) (Martín and Langella 2019). As described in Table 17.2, the probiotics available in the market contain microorganisms mostly belonging to genera *Lactobacillus* and *Bifidobacterium*. Nevertheless, there are also some members of *Bacillus* and *Streptococcus* for bacteria and yeast strains belonging to the genus *Saccharomyces* (Gomes et al. 2017). These classical probiotic strains have a long history of use, and they are well-characterized regarding to safety point of view (Martín and Langella 2019). However, in some cases, strains display limited effects on the human gut microbiota evoking the need for a better selection of microbial strains and improvement in the development of their delivery systems (Neef and Sanz 2013).

In the last years, several bacterial species involving genera other than *Lactobacillus* and *Bifidobacterium* with promising outcomes in the treatment and prevention of diverse metabolic and inflammatory diseases have been proposed as potential next-generation probiotics, NGPs for short (Almeida et al. 2019). Nonconventional candidate strains include *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Eubacterium hallii* (recently reclassified as *Anaerobutyricum hallii*), *Bacteroides fragilis*, *Bacteroides uniformis*, and members of the Clostridia clusters IV, XIVa, and XVIII (El Hage et al. 2017; Almeida et al. 2019). These novel candidates have been mainly identified based on

Table 17.2 Probiotic microorganisms available in the market

Lactobacillus genus	*Bifidobacterium* genus	Other microorganisms
L. acidophilus	*B. adolescentis*	*Bacillus coagulans*
L. buchneri	*B. animalis*	*Bacillus subtilis*
L. brevis	*B. animalis* subsp. *lactis*	*Streptococcus thermophilus*
L. casei	*B. catenulatum/ pseudocatenulatum*	*Saccharomyces cerevisiae* variant *boulardii*
L. crispatus	*B. bifidum*	
L. delbrueckii subsp. *bulgaricus*	*B. breve*	
L. delbrueckii subsp. *lactis*	*B. lactis*	
L. fermentum	*B. longum*	
L. helveticus		
L. paracasei		
L. paracasei subsp. *paracasei*		
L. pentosus		
L. plantarum		
L. reuteri		
L. rhamnosus		
L. salivarius		
L. salivarius subsp. *salivarius*		

Adapted from Gomes et al. 2017

comparative analysis of microbiota compositions between both healthy and unhealthy subjects (Qin et al. 2012; Lopez-Siles et al. 2015; Martín and Langella 2019). In contrast to classical probiotics, the NGPs are not yet available in the common market. The introduction of these NGPs, also termed as live biotherapeutic products (O'Toole et al. 2017), in the nutraceutical/pharmaceutical market requires a full assessment of safety parameters and an in-depth characterization of their beneficial effects on the host (Brodmann et al. 2017; El Hage et al. 2017). Moreover, the development of delivery vehicles for these novel probiotics are urgently needed due to their stringent survival conditions. These delivery systems should simultaneously confer greater microbial viability and high efficacy of probiotic action and should be safe for human use (Almeida et al. 2019). Thus, NGP introduction and implementation in the market demands a close interaction between research/clinical institutions, pharmaceutical/food industries, and regulatory agencies.

17.3.3 Alternative Strategies to Promote Health Benefits: Knowing Other Biotics

The current interest in actively improving the host health via modulation of the intestinal microbiota is progressively becoming more prominent. Traditionally, this has been an action endeavored by the use of probiotics. However, data suggests that pre-, syn-, and postbiotics also exhibit as correspondingly important sources to positively impact human wellness, by improving gut microbiome composition and stimulating optimal immune system function (Miniello et al. 2015).

Prebiotics is a term that encompasses a wide range of substrates that are selectively utilized by friendly host microorganisms, conferring health benefits (Gibson et al. 2017). Indeed, prebiotics selectively stimulates beneficial microorganisms present in the intestinal tract, affecting their fermentation activity and subsequently influencing the short-chain fatty acid (SCFA) level, which leads to positive health effects (Van-Den-Abbeele et al. 2013; Sivieri et al. 2014). Commonly, most prebiotics used in human nutrition include fructooligosaccharides, galactooligosaccharides, inulin, xylooligosaccharides, lactitol, lactosucrose, lactulose, soy oligosaccharides, and transgalactooligosaccharides (Markowiak and Slizewska 2017). However, several noncarbohydrate structures such as polyphenols, long chain polyunsaturated fatty acids, minerals, or vitamins has also been classified as prebiotic (Steinert et al. 2016). In fact, prebiotics can be naturally present in certain foods since some dietary fibers are prebiotics. However, they may also be added to foods with a purpose to enhance their nutritional and health value (Markowiak and Slizewska 2017). For the classification of a food ingredient as prebiotic, the fulfillment of five criteria, namely, resistance to digestive processes, selective fermentation by potentially beneficial bacteria in the colon, beneficial

effect on the health of the host, selective stimulation of probiotics, and stability to food processing treatments, is required (Wang 2009). In a sense, pro- and prebiotics are, therefore, interdependent, in which prebiotics encourage probiotics intestinal population to flourish. The recognition of this close relationship led to the development of synbiotic products. According to Martín and Langella (2019), synbiotics are "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare." Due to the high number of possible combinations involving prebiotics and probiotics, the synbiotics products seem to be a compelling approach for modulation of human gut microbiota (Markowiak and Slizewska 2017). Thus, the health benefits claimed by synbiotics consumption by humans include increased abundance of lactobacilli and bifidobacteria (Van-Zanten et al. 2014), improvement of clinical course of cirrhotic patients (Fukui 2015), attenuation of inflammatory markers in patients with nonalcoholic fatty liver disease (Eslamparast et al. 2014), and improvement of symptoms associated to atopic dermatitis in children (Ibáñez et al. 2018).

Importantly, recent evidences suggest that bacterial viability is not a mandatory requirement to attain the health-promoting effects since not all mechanisms, nor clinical benefits, are directly associated to the viability of probiotic microorganisms (Choi et al. 2006; Cicenia et al. 2014). Such understanding has been cementing postbiotics as an emerging field of research, revolutionizing probiotic science. Postbiotics, also known as either metabiotics, biogenics, metabolites, or cell-free supernatants, are soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis (Cicenia et al. 2014; Aguilar-Toalá et al. 2018). Therefore, postbiotics can comprehend enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell surface proteins, vitamins, plasmalogens, SCFAs, and organic acids, and other complex biomolecules, and they are known to be important in regulating intestinal biological activity (Aguilar-Toalá et al. 2018). These compounds have drawn attention due to their clear chemical structure, dose and safety parameters, long shelf life, and the content of various signaling molecules which may have anti-inflammatory, immunomodulatory, anti-obesogenic, antihypertensive, hypocholesterolemic, antiproliferative, and antioxidant effects (Shenderov 2013; Aguilar-Toalá et al. 2018).

17.4 Encapsulation of Probiotics

Encapsulation has arisen as a strategy to mitigate some of the limitations to the use of probiotics in food and therapeutic applications. Encapsulation of bioactive components has been used for a long time in many applications in the food and pharmaceutical industries: preventing biodegradation and undesirable chemical reactions;

masking flavors, colors, and odors; improving solubility; and providing sustained and controlled release (Nedovic et al. 2011; Santiago and Castro 2016). Probiotic encapsulation has been mainly used to protect the cells against an adverse environment rather than a controlled release. The term encapsulation is related to the entrapment of several substances (active ingredients) within another material (encapsulant). Depending on the method and materials used, we may obtain microcarriers, with sizes ranging from 1 to 1000 μm, or nanocarriers with sizes ranging from 1 to 1000 nm (Quintanilla-Carcavajal et al. 2010; Suganya and Anuradha 2017). However, this size threshold is controversial and is not unanimous; for instance, the EFSA (EFSA Scientific Committee 2011) refers to NPs as engineered nanomaterials that have at least one dimension in the range of 1–100 nm. As probiotic cells typically have sizes ranging from 1 to 5 μm, it is not possible to encapsulate them in NPs. Particle size is also important, particularly for food applications, as it may negatively affect the sensory properties of the food product (Champagne and Fustier 2007; Heidebach et al. 2012). For instance, Hansen et al. (2002) reported a particle size below 100 μm to avoid a "gritty" sensation when consumed. However, particles with larger diameters, and thus with higher volume-to-surface ratio, may have an increased probiotic protective effect (Anal and Singh 2007). A proper balance between sensory and protective properties must be taken into consideration before application of probiotic microparticles in food products. Another difficulty of probiotic encapsulation is the fact that they must be kept alive as their health effects are dependent on their viability after consumption. This means that materials and methodologies used for probiotic microparticle production should be carefully evaluated.

In the following sections, a brief and updated overview of the main materials and techniques used for probiotics microencapsulation is presented. In Table 17.3, a selection of recently published works (over the last 4 years) dealing with probiotic encapsulation is presented.

17.4.1 Encapsulating Materials

The choice of encapsulation materials is of extreme importance to ensure the wanted protection as well as production efficiency and compatibility with the desired application (Chen et al. 2017a). For food applications polysaccharides, proteins and lipids have been the obvious choices and, thus, the most used encapsulation materials.

17.4.1.1 Polysaccharides

Several polysaccharides such as alginate, pectin, chitosan, gellan gum, k-carrageenan, starch, and xanthan gum have been used for probiotic encapsulation (Martín et al. 2015; Kavitake et al. 2018; Kwiecien and Kwiecien 2018; Călinoiu et al. 2019).

Table 17.3 A selection of studies dealing with probiotic encapsulation during 2016–2019

Probiotic strain	Encapsulant material	Encapsulation technique	Particle size (μm)	Main achievements	Reference
Bacillus coagulans BC	Alginate, chitosan	LbL	–	Improved survivability in vitro simulated gastrointestinal conditions	Anselmo et al. (2016)
L. plantarum NCDC 012, *L. casei* NCDC 297 and *L. brevis*	β-Glucan	Emulsification	–	Improved survivability in vitro simulated gastrointestinal conditions and under thermal treatments	Shah et al. (2016)
B. bifidum BB01	Xanthan, chitosan	Extrusion	–	Improved survivability in vitro simulated gastrointestinal conditions and increased storage stability in yogurt (21 days storage at 4 °C and 25 °C)	Chen et al. (2017b)
L. plantarum ATCC 13643	Carboxy-methyl cellulose, k-carrageenan	Extrusion	–	Improved survivability in vitro simulated gastrointestinal conditions	Dafe et al. (2017)
L. acidophilus ATCC 4356	Chitosan, phytic acid	Extrusion (electrostatic)	1300–1500	Improved survivability during storage and exposure to acid	Kim et al. (2017)
L. casei DSM 20011, *L. reuteri* DSM 20016 and *L. delbrueckii* subsp. *bulgaricus* DSM 2008)	Alginate	Extrusion (vibration nozzle)	600–800	Improved survivability under exposure to low acid conditions	Olivares et al. (2017)

(continued)

Table 17.3 (continued)

Probiotic strain	Encapsulant material	Encapsulation technique	Particle size (μm)	Main achievements	Reference
L. paracasei A13 and *L. salivarius* subsp. *salivarius* CET 4063	Alginate	Emulsion with high-pressure homogenization	<100	Improved survivability in vitro simulated gastrointestinal conditions and production of fermented milk with improved functionality and with enhanced sensory properties	Patrignani et al. (2017)
L. acidophilus TISTR 1338	Alginate, egg albumin, stearic acid, and cassava starch	Electrospraying and fluidized bed coating	450	Improved survivability under moist heat treatment (70 ± 0.5 °C, 100% relative humidity, 30 min)	Pitigraisorn et al. (2017)
B. animalis subsp. *lactis* BB12	Full-fat goat's milk, inulin, or oligofructose	Spray-drying	–	Improved survivability in vitro simulated gastrointestinal conditions and under thermal treatments	Verruck et al. (2017)
L. plantarum ATCC 8014	Alginate, Chitosan	Electrospraying	300–400	Improved survivability during storage and exposure to acid	Zaeim et al. (2017)
L. plantarum CECT 220 and *L. casei* CECT 475	Soybean protein concentrate, maltodextrin, and oligofructose-enriched inulin	Coacervation followed by spray-drying	11	Improved survivability in vitro simulated gastrointestinal conditions and storage (25 °C, >100 days)	Gonzalez-Ferrero et al. (2018)
L. acidophilus La-5	Gum Arabic, inulin, hi-maize, and trehalose	Spray-drying	6.7–19.3	Improved survivability in vitro simulated gastrointestinal conditions and thermal resistance and increased storage stability (120 days, 25 °C)	Nunes et al. (2018)

(continued)

Table 17.3 (continued)

Probiotic strain	Encapsulant material	Encapsulation technique	Particle size (μm)	Main achievements	Reference
L. acidophilus LA5	Alginate, gelatin, FOS	Extrusion (atomization)	–	Improved survivability in vitro simulated gastrointestinal conditions and during the storage in yogurt	Silva et al. (2018a)
L. *acidophilus* LA3 and *B. animalis* subsp. *lactis* BLC1	Vegetable fat, gum Arabic, gelatin	Spray chilling and electrostatic interaction	79–84	Improved survivability in vitro simulated gastrointestinal conditions as well as under different stress conditions (low pH, high level of sucrose, and NaCl)	Silva et al. (2018b)
L. casei 39392	Whey protein	Electrospraying and transglutaminase cross-linking	3.1	Improved survivability in vitro simulated gastrointestinal conditions and storage (20 °C, 120 days)	Alehosseini et al. (2019)
B. longum DD98	Alginate, chitosan	Emulsification	190	Improved survivability in vitro simulated gastrointestinal conditions, under thermal treatments and storage (25 °C, 180 days)	Ji et al. (2019)
B. longum	Soy protein isolate and carrageenan	Complex coacervation	–	Improved viability during storage (4 °C), pasteurization (85 °C for 5, 10 and 30 min) and in vitro dynamic gastric and intestinal digestion	Mao et al. (2019)

(continued)

Table 17.3 (continued)

Probiotic strain	Encapsulant material	Encapsulation technique	Particle size (μm)	Main achievements	Reference
L. paracasei BGP1 and *L. rhamnosus* 64	Vegetable fat, gum Arabic, gelatin	Complex coacervation	80	Improved stability in the presence of salt and in simulated gastrointestinal conditions and encapsulated microorganisms maintained their viability and functionality during storage (120 days)	Matos Jr et al. (2019)
L. plantarum	Gelatin, gum Arabic	Double emulsification and complex coacervation	66–106	Improved survivability in vitro simulated gastrointestinal conditions and storage (8 °C and –18 °C, 45 days)	Paula et al. (2019)
L. acidophilus	Alginate, rice bran, inulin, Hi maize	Extrusion (atomization)	80–118	Improved survivability in vitro simulated gastrointestinal conditions. Alginate, rice bran and Hi-maize microparticles maintained viable probiotics for 120 days. At −18 °C, only inulin remained stable for 120 days. At 7 °C, rice bran and inulin preserved viable probiotics over 120 days of storage	Poletto et al. (2019)
S. boulardii CGMCC 10381and *E. faecium* CGMCC 2516	Alginate	Emulsification	300–500	Improved survivability in vitro simulated gastrointestinal conditions and under thermal treatments	Qi et al. (2019)
B. bifidum	Alginate, zein	Extrusion	1210–1720	Improved survivability in vitro simulated gastrointestinal conditions and storage (4 °C)	Riaz et al. (2019)

Alginate is probably the most extensively used biopolymer for encapsulation. It is an anionic, linear heteropolysaccharide composed of D-mannuronic and L-guluronic acids. The composition and the sequence of L-guluronic and D-mannuronic acids vary widely, depending on the source and, thus, its functional properties. In the presence of divalent cations, alginate forms a gel with an "egg-box" structure (Martín et al. 2015). Beads can be formed by dripping a mixture of a sodium alginate solution with a cell suspension into a solution containing cations (usually Ca^{2+} in the form of $CaCl_2$). Alginate microparticles can be obtained by an external or internal gelation process. In the first case, the microparticles are produced by the formation of a water-in-oil emulsion, usually stabilized by surfactants, such as Tween 80. The alginate is then gelled by the addition of calcium containing solution to the emulsion. In the internal gelation process, alginate is first mixed with an insoluble calcium salt (most often calcium carbonate) before the formation of a water-in-oil emulsion. The addition of an organic acid (most often acetic acid) to the emulsion releases calcium ions (and carbonic acid) which will initiate alginate gelation. Although probiotic bacteria can be well encapsulated into the alginate particles with high viability, the structure of the cross-linking polymers formed by divalent cations are turned out to be porous, which cause the easy entry and exit of H^+ and other detrimental substances leading to the damage of cells (Liu et al. 2019). Other disadvantages are related to the scaling-up of the process that is difficult. These drawbacks can be overcome by combining alginate with other polymers or by coating capsules with other compounds or using different additives for structural modification of the alginate (Kavitake et al. 2018). Silva et al. (2018a) produced microbeads of alginate-gelatin and alginate-gelatin-fructooligosaccharides by external gelation, to improve the viability of *L. acidophilus* LA5 when exposed to the gastrointestinal tract and during storage when added to yogurt. Riaz et al. (2019) used zein as coating material of alginate microbeads to improve survival during gastric transit and storage of *B. bifidum.* Other researchers used chitosan as a coating material. Ji et al. (2019) used chitosan-coated alginate microcapsules obtained by emulsification and internal gelation method to extend the viability of *B. longum* DD98. Anselmo et al. (2016) produced chitosan and alginate microparticles using an LbL (layer-by-layer) encapsulation process to increase *Bacillus coagulans* resistance against acidic and bile salt insults.

Chitosan is also an extensively used biopolymer for probiotic encapsulation used mainly in combination with other polysaccharides (for instance, alginate as described above). It is an aminopolysaccharide derived from chitins, composed of β-(1,4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. The main advantages of chitosan coating are a unique cationic character, high biocompatibility, nontoxicity, and biodegradability (Călinoiu et al. 2019). However, it has antimicrobial properties, and therefore, it is not suitable to be used as sole encapsulant material for creating probiotic delivery systems (Kwiecien and Kwiecien 2018).

Other polysaccharides such as inulin, fructooligosaccharides, and other dietary fibers have also been increasingly used not only for their prebiotic properties but also for their protecting capabilities (Table 17.3).

17.4.1.2 Proteins

Several food proteins have been used alone or combined with other compounds to encapsulate probiotics. Proteins usually have good emulsifying properties, and aqueous solutions of most proteins have a relatively low viscosity, even at high concentrations. This facilitates the formation of microparticles with dense gel network that provides a substantial buffering capacity, thereby supporting the idea of a protective barrier between the active ingredients and the surrounding environment (Heidebach et al. 2012). Gelatin, milk proteins, and vegetable proteins (soy, pea, cereal proteins) are the most commonly used to encapsulate probiotics.

Gelatin is a protein derived by partial hydrolysis of collagen of animal origin. It has a very special structure and versatile functional properties and forms a solution of high viscosity in water, which sets to a gel during cooling (Gbassi and Vandamme 2012). Due to its amphoteric nature, it is an excellent candidate for cooperation with anionic polysaccharides such as gellan gum (Burgain et al. 2011). Nawong et al. (2016) developed and characterized novel food-grade gelatin–maltodextrin microparticles cross-linked with transglutaminase that protects the encapsulated *Lactobacillus* spp. under simulated gastrointestinal conditions. Paula et al. (2019) used gelatin and gum Arabic for the encapsulation of *L. plantarum* by a dual process combining double emulsification followed by complex coacervation. The formed microparticles maintained the viability of *L. plantarum* cells during storage for 45 days at 8 °C and −18 °C and high survivability in simulated gastrointestinal conditions.

Milk proteins (caseins and whey proteins) are remarkable encapsulation materials because of their biocompatibility and their gel-forming ability like gelatin under suitable conditions (Kavitake et al. 2018). Due to their structural and physicochemical characteristics, they are suitable as natural vehicles in probiotic encapsulation (Livney 2010; Abd El-Salam and El-Shibiny 2015). Microencapsulation of *L. paracasei* and *B. lactis* in casein hydrogels obtained by transglutaminase or rennet cross-linking increased the encapsulation efficiency and the viability of probiotics in simulated gastric condition as well as during freeze-drying and storage (Heidebach et al. 2012). Alehosseini et al. (2019) used electrospraying technique for the encapsulation of *L. casei* in transglutaminase cross-linked whey protein concentrate/whey protein isolate (WPI) matrix. Spherical water-resistant capsules with an average diameter of 3.09 μm were obtained with high encapsulation efficiency and high viability of *L. casei* under the simulated gastrointestinal conditions.

Vegetable proteins are becoming increasingly important to the food industry as a replacement for animal-derived proteins. The application of all-plant-based matrices for encapsulation of organisms could expand probiotic use in food products and markets that restrict the use of animal-derived proteins as an encapsulating material due to low cost, renewability, functionality, and religious, moral, or dietary preferences (Wang et al. 2015). Gonzalez-Ferrero et al. (2018) reported the capacity of soybean protein concentrate for encapsulating probiotics in biodegradable microparticles prepared by coacervation and dried by spray-drying. The resulting microparticles, of about 11 μm, showed a spherical matrix in which bacteria were

uniformly distributed. The encapsulation of probiotics increased significantly their stability during storage under controlled conditions (25 °C/60% RH) and enhanced significantly in vitro gut resistance. Mao et al. (2019) investigated the roles of soy protein isolate and carrageenan coacervates in microencapsulating *B. longum*. The coacervates were effective in improving the viability of probiotics during storage (4 °C), in vitro gastrointestinal digestion, and pasteurization (85 °C). Pulse proteins represent an attractive alternative to soy due to their non-genetically modified status and low risk of allergenicity. Wang et al. (2015) investigated the use of legume proteins from pea, faba bean, and lentil combined with small amounts of alginate for the microencapsulation of *B. adolescentis* ATCC 15703 using an emulsion-based technique. Except for the lentil protein formulation, all microparticles were approximately 20 μm in diameter. Pea protein microparticles provided the greatest protective effect for *B. adolescentis* cells in simulated gastric juice. Varankovich et al. (2015) studied the suitability of pea protein isolate mixed with sodium alginate, iota-carrageenan, or gellan gum, as protective materials for acid-sensitive *B. adolescentis* 15703 under simulated stomach conditions. Overall, the increase in survivability of the probiotics was similar to all types of capsules. Following a temporal rat feeding study with the test bacterium encapsulated in pea protein isolate with alginate, *B. adolescentis*-specific PCR and qPCR analyses confirmed the presence of DNA from this species in rat feces, but only during the period of capsule intake.

17.4.1.3 Lipids

Lipidic materials have been proposed to be used as potential encapsulation matrices because diffusion of acids, water, and oxygen across lipid particle membranes is limited (Lahtinen et al. 2007; Pedroso et al. 2012). However, compared to other encapsulant materials, lipids have been much less explored for probiotic encapsulation. Lipid-based encapsulation usually involves the dispersion of the probiotics in molten fat and subsequent cooling. The melting temperature of the chosen fat material is of crucial importance as it can negatively influence probiotic survivability during the encapsulation process. Pedroso et al. (2012) used an interesterified fat with palm and palm kernel, which has a melting point of 47.5 °C, to encapsulate *B. lactis* BI-01 and *L. acidophilus* LAC-04 using spray-chilling and found that there was no loss in cell viability in both probiotic bacteria. Similar results were also found by Pedroso et al. (2013) using cocoa butter (melting point of 36.5 °C) to encapsulate the same probiotic microorganisms. Okuro et al. (2013) co-encapsulated *L. acidophilus* LAC-04 with prebiotics (inulin or polydextrose) in solid lipid microparticles using spray chilling technology and an interesterified fully hydrogenated palm and palm-kernel oil (melting point of 43.34 °C) as encapsulant matrix. More recently, Amakiri et al. (2015) also developed lipid-based symbiotic particles containing *B. longum* LMG 13197 using glyceryl dipalmitostearate (melting point 57 °C) and inulin. These authors used a double-emulsion W/O/W method followed by freeze-drying. This method does not require the melting of the fat material but

involves the use of organic solvents (dichloromethane) which can limit the use of these microparticles in food applications.

17.4.2 Microencapsulation Methods

As mentioned above, probiotics present two sets of problems when considering encapsulation: their size (typically ranging from 1 to 5 μm diameter), which excludes nanotechnologies, and the fact that they must be kept alive. Moreover, the size of microcarriers may also be another limitation depending on the desired application. Thus, the selection of the encapsulation technology for probiotics needs to consider these and other (cost and operating efficacy, for instance) aspects. The most commonly reported techniques for probiotics encapsulation (conventional techniques) in the scientific literature are extrusion, emulsification, and spray-drying. However, other techniques such as electrospinning, spray-chilling, LbL, and complex coacervation have been increasingly used for probiotic encapsulation (emerging techniques).

17.4.2.1 Conventional Encapsulation Techniques

Extrusion

The basic extrusion technique involves the dripping of a hydrocolloid solution (most often alginate) containing probiotic bacteria through a syringe needle into a hardening solution (most often $CaCl_2$). Owing to its simplicity, low cost, gentle conditions, and high cell viability, the extrusion method is one of the most popular methods that is widely used to encapsulate probiotics. The main disadvantages of this technology are the process duration, the difficulty of scale-up (Burgain et al. 2011), and the impossibility in producing capsules smaller than 500 μm by a conventional dropwise method (Krasaekoopt et al. 2003). To obviate these drawbacks, variations of the basic technique were developed using spray systems, such as vibrating nozzles, air-atomizing nozzles, and electrostatic and spinning-disk atomization (Chavarri et al. 2012; Ramos et al. 2018). *Lactobacillus acidophilus* ATCC 4356 encapsulated in a chitosan/phytic acid matrix obtained by ionic gelation with electrostatic extrusion showed improved survival rate under refrigerated storage and simulated gastric conditions (Kim et al. 2017). Similar results were also found by Poletto et al. (2019) using alginate microparticles added with prebiotics (inulin, rice bran, or Hi-maize) by extrusion/external gelation using an air-atomization nozzle. Alginate microcapsules obtained by extrusion, with or without double coating, revealed to be suitable to protect *L. paracasei* L26 incorporated in low pH juice fruits since viable cells were approximately 9 log cfu/g after 50 days of storage at 5 °C (Rodrigues et al. 2012).

Nowadays, the extrusion method presents a vast diversity of industrial equipment able to be adapted to create particles from different mixtures of polymers and cross-linkers. Furthermore, these equipment are also able to obtain particles' sizes that are not attainable with conventional procedures at a laboratorial scale (Ramos et al. 2018).

Emulsion

In this method, a small volume of an aqueous hydrocolloid probiotic mixture (discontinuous phase) is emulsified into a larger volume of vegetable oil (continuous phase). Once a water-in-oil emulsion has been formed, the dispersed hydrocolloid-cell mixture must be insolubilized to form small particles within the oil phase. For alginate hydrogels, this solubilization can be achieved by external or internal gelation. The diameter of microparticles is dependent on the concentration and viscosity of the hydrocolloid solution and its agitation speed (Kavitake et al. 2018). In contrast to extrusion, the emulsion-based technique is easier to scale-up with high bacterial survival rate and producing capsules with a smaller diameter (ranging between 25 and 2000 μm) which are the main added advantages. In turn, its main disadvantage is large size range and shape of microparticles, difficult separation from the different phases (risk of damaging the particles), and higher cost for performance owing to the usage of vegetable oil (Kavitake et al. 2018; Ramos et al. 2018). Microparticles of *B. lactis* BB12 formed by emulsification/internal gelation using alginate as an encapsulating agent provided effective protection under simulated gastrointestinal conditions and 120 days of frozen storage (Holkem et al. 2017).

Spray-Drying

Spray-drying is a well-established process in the food industry to convert liquids into dry powders. This technology has been utilized on probiotic cells with the purpose of not just simply drying, but also as an encapsulation procedure. It consists in spraying the liquid feed in fine droplets (10–150 μm) that are directed into a flow of hot and dry air (usually 150–250 °C) (Huang et al. 2017). The increase in the air-liquid interface area subsequent to spraying dramatically becomes greater the drying kinetics, and it is commonly admitted that drying occurs within a few seconds (Huang et al. 2017). The main disadvantage of this technology is due to the high temperature, osmotic stress, dehydration, and oxygen exposure conditions applied during the process that can result in the damage of probiotic cells. However, proper adjustment and control of the processing conditions such as the inlet and the outlet temperatures or the addition of thermal protectants (such as trehalose or prebiotics) can achieve viable encapsulated probiotics with a desired particle size distribution (Burgain et al. 2011). Microparticles containing gum Arabic mixed with inulin, himaize, or trehalose were produced through spray-drying to encapsulate *L.*

acidophilus La-5 by Nunes et al. (2018). The formulations containing trehalose and hi-maize were the encapsulating matrices with higher protective capacity relative to, respectively, thermal and simulated gastrointestinal conditions. Whey protein microencapsulation via spray-drying, with or without L-cysteine-HCl of *L. acidophilus* Ki, *L. paracasei* L26, and *B. animalis* BB12, was performed and stored up to 6 months at 5 °C and 22 °C under different values of relative air humidity and oxygen (Rodrigues et al. 2011); *L. paracasei* L26 was the least susceptible to storage conditions presenting values above 10^6 cfu g^{-1} by 180 days at 22 °C, irrespective of relative humidity and the presence/absence of oxygen and L-cysteine.

17.4.2.2 Emerging Encapsulation Techniques

Spray-Chilling

Spray-chilling (or spray cooling or spray congealing) is similar to spray-drying with respect to droplet formation. Spray-chilling involves the preparation of a solution, dispersion, or emulsion containing the active agent and a molten matrix (typically a lipid), which is then atomized into a chamber where cold air is injected (Okuro et al. 2013). Owing to its low cost, use of low temperature, and easy to scale up nature, spray-chilling is considered a suitable technology for the encapsulation of food ingredients (Liu et al. 2019). However, some technological disadvantages still exist, such as low encapsulation efficiency and the possibility of the expulsion of the active ingredient from the matrix during storage.

Electrospraying

Electrospraying is also known as electro-hydrodynamic atomization which is based on the principle of liquid atomization using electrical forces. The liquid flowing out of a capillary nozzle, at high electric potential, is forced by the electric field to be dispersed into fine droplets (Coghetto et al. 2016a). The size of electrospray droplets can range from hundreds of micrometers down to tens of nanometers. The main advantages of this technique are the fact of operating under mild conditions, simplicity, and the possibility of large-scale production. Coghetto et al. (2016b) used the electrospraying technique to microencapsulate *L. plantarum* BL011 in sodium alginate or sodium alginate-citric pectin matrixes. The authors demonstrated the efficiency of this technique to increase the cell survival of *L. plantarum* under simulated gastrointestinal conditions and under refrigeration when compared to free cells. Similar results were obtained by Zaeim et al. (2017) who also used the electrospraying technique to microencapsulate *L. plantarum* ATCC 8014 in Ca-alginate/chitosan hydrogel.

Layer by Layer

The LbL technique involves the alternative adsorption of oppositely charged materials on surfaces, thereby providing a system with tunable properties. The thickness, permeability, strength, and morphology of the layers can be tailored with precision (by altering the pH, ionic strength, wall materials), providing an ambiance with the desired properties (Priya et al. 2011). Unlike other methods, LbL is unique because each individual cell in suspension is coated sequentially, affording complete encapsulation (Priya et al. 2011). The survival rate of *L. acidophilus* encapsulated through LbL self-assembly of the polyelectrolytes chitosan and carboxymethyl cellulose was enhanced in simulated gastrointestinal conditions (Priya et al. 2011). The higher resistance of the encapsulated microorganism was attributed to the impermeability of polyelectrolyte nanolayers to pepsin and pancreatic enzymes. Moreover, it also reduced viability losses of the microorganism during freezing and freeze-drying. Anselmo et al. (2016) reported a LbL method for the encapsulation of *Bacillus coagulans* to protecting it from gastrointestinal challenges while facilitating both mucoadhesion and direct growth on intestinal surfaces.

Coacervation

This technique exploits the phase separation of one or more incompatible polymers from the initial coating polymer solution under specific temperature, pH, or composition of the solution (Coghetto et al. 2016a). The incompatible polymer is added to the coating polymer solution, and the dispersion is stirred. Changes in the physical parameters result in the separation of incompatible polymer and deposition of dense coacervate phase surrounding the core material (probiotic cells) resulting in the production of microparticles. The main advantages of this technique are the fact of operating under mild conditions (absence of use of high temperatures or organic solvents), simplicity, low cost, and the possibility of incorporating a large amount of microorganisms in relation to the encapsulant (Chavarri et al. 2012). However, the scale-up of coacervation is difficult, since it is a batch process that yields coacervate in an aqueous solution. This implies that an additional drying process (such as spray-drying) may be necessary, which can be harmful to the probiotic cells.

17.5 Nanotechnology Applied to Probiotic: "How to Go Smaller"

Nanotechnology has been considered as an advantage for medicine, through the development of nanocarriers and/or devices at scales less than 100 nm (Caneus 2017). Application of nanotechnology in designing nanoprobiotics has gained tremendous potential worldwide in the field of nutraceuticals and is expected to grow

rapidly in the future, in areas such as agriculture and food industry (Pathak and Akhtar 2019). The application of nanotechnology in the Agriculture and Food sectors are relatively recent compared with its use in drug delivery and pharmaceutical sector (Sozer and Kokini 2009). The basic of probiotics nanotechnology application is currently in the development of nano-encapsulated probiotics, aiming to offer an improvement on the taste, texture, and consistency of nanostructured food ingredients (Chau et al. 2007). However, the application of nanotechnology in food requires some precaution, namely, its impact on the environment and human health. Currently, there are no regulations that specifically control or limit the production of nanosized particles, and this is mainly owing to a lack of knowledge about the risks (Sozer and Kokini 2009). Nanoencapsulation is a technology able to entrap bioactive compounds using different techniques: nanoemulsification, nanocomposite, and nanostructuration developing controlled release nanocarriers (Sekhon 2010; Song et al. 2012). Nanoencapsulation is desirable to develop designer probiotic bacterial preparations—nanoprobiotics—that could be delivered to certain parts of the gastrointestinal tract where they interact with specific receptors (Sekhon 2010). The designed nanoprobiotics may work as de novo vaccines by modifying the immune response, being effective in supplementing various therapies such as irritable bowel syndrome and gastrointestinal infections. Nanosized materials have a distinctive potential to enhance the bioavailability or functionality of nutrients as well as ingredients. As previously mentioned, probiotic size ranges from 1 to 5 μm of diameter, making its nanoencapsulation quite a challenge. However, various nanocarriers are available (Fig. 17.3), and these have been effectively researched to formulate the incorporation of probiotic bacteria. Table 17.4 highlights some reports on nanoencapsulation demonstrating the importance of these technologies in probiotics delivery.

Encapsulation of probiotics into microcapsules or microgels can be used to enhance the viability and stability of probiotics in food and the gastrointestinal tract (as discussed above). However, the porosity is a problem, and so small ions and molecules in the surrounding solutions, such as bile salts or digestive enzymes,

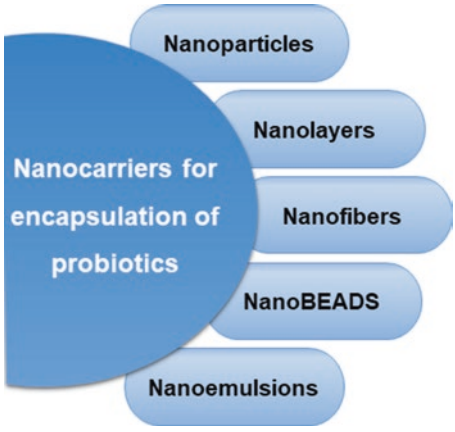

Fig. 17.3 Different nanocarriers for encapsulation of probiotics

Table 17.4 Delivery of encapsulated probiotics via nanocarriers

Nanocarriers	Delivered probiotics or other microorganism	Main achievements	Reference
Nanoparticles	*Salmonella* spp.	Inhibition of tumor growth by efficient oral delivery of vascular endothelial growth factor receptor 2 (VEGFR2) with nanoparticle-coated bacteria vectors due to angiogenesis suppression in the tumor vasculature and tumor necrosis	(Hu et al. 2015)
	Salmonella typhimurium	*Salmonella* surface with the sucrose-linked gold nanoparticles and bacteria biotinylated to link the streptavidin-conjugated fluorophores; biotin concentration was increased on the membrane surface, leading to an improvement in the tumor destruction	(Kazmierczak et al. 2015)
	L. plantarum	Selenium nanoparticles to be employed as an immunomodulating agent; mice treated with selenium nanoparticle-enriched *L. plantarum* induced a reduction of the tumor and increased the mice survival rate compared with the mice treated with free bacteria	(Yazdi et al. 2012)
	L. plantarum	*L. plantarum* PTCC1058 was used for the intracellular synthesis of tellurium nanoparticles; the results demonstrated the potential of *L. plantarum* with deposited tellurium nanoparticles and bacteria devoid of nanoparticles in the reduction of serum cholesterol in mice fed and gavage by a single dose of cholesterol. Bacteria with deposited nanoparticles were more effective than bacteria without nanoparticles in decreasing triglyceride levels	(Mirjani et al. 2015)
Nanolayers	*Allochromatium vinosum*	Development of LbL nano self-assembled coated bacteria by polyelectrolyte combinations showed that surface charge did affect neither sulfide uptake nor the contact formation between the cells and solid sulfur	(Franz et al. 2010)
	L. acidophilus	The survival of encapsulated bacteria in the gastrointestinal tract was higher than nonencapsulated bacteria; the polyelectrolyte coating also served to reduce viability losses during freezing and freeze-drying	(Priya et al. 2011)

(continued)

Table 17.4 (continued)

Nanofibers	*L. acidophilus*	Electrospinning was a capable way for the stable solid formulation of *L. acidophilus*, increasing long-term stability	(Nagy et al. 2014)
	L. plantarum	The feasibility of encapsulating bacteriocins and lactic acid bacteria into spun nanofibers was demonstrated	(Heunis et al. 2010)
	L. acidophilus	Bacteria were incorporated into the spinning solution to produce a nanofiber-encapsulated probiotic; exhibited good bacteria survivability (78.6–90%) under electrospinning conditions and retained viability at refrigeration temperature during the 21 days storage	(Fung et al. 2011)
	B. animalis BB12	Prepared nanofiber by electrospinning for encapsulating bifidobacterial strain, in order to improve viability and stability; exhibited an enhancement of viability up to 40 days at room temperature and for 130 days under refrigerated conditions in comparison to non-encapsulated bacteria	(López-Rubio et al. 2009)
	Bacillus spp.	Clinical isolate strain was incorporated into nanofibers to fight a periodontal pathogen bacterium; nanofibers increased the viability of probiotic and storage time. Probiotic was released from nanofibers, and the antimicrobial activity against periodontal pathogen bacteria was confirmed	(Zupančič et al. 2018)

could easily diffuse into microcapsules, which may lead to degradation of the encapsulated probiotics (Zhang et al. 2017). One strategy to overcome this problem is the addition of inorganic NPs. Yao et al. (2018) encapsulated *Pediococcus pentasaceus*Li05 in alginate-gelatin microgels in the absence and presence of magnesium oxide NPs. They demonstrated that probiotic encapsulated in MgO-loaded microgels was more stable than free bacterial cells or those encapsulated in microgels alone. The addition of MgO NPs improves the viability of the probiotics, which can be attributed to its antiacid effects. NPs may lead to enhanced probiotic viability by filling pores inside the microgels, which may have inhibited the ability of oxygen and hydrogen ions to access the probiotics, because the MgO NPs are able to neutralize the hydrogen ions in the gastric fluids, reducing acid-induced degradation of the probiotics. These NPs can act as a matrix fortifier to fill in the pores generated during freeze-drying. In the intestinal fluids, the presence of the MgO NPs also improves the viability of the probiotics, which may again have been because these filled in the pores in the hydrogel matrix inside the microgels, leading to decrease of bile salt diffusion into the microgels and damage of the probiotics (Yao et al. 2018).

Probiotic bacteria may be employed during the synthesis of NPs. Metallic NPs have been a wide broad spectrum of application, since engineering (optical or

tissue), cosmetic or drug application, as well as nanodevices (Akhtar and Pathak 2017). The invention of Domínguez Vera et al. (2014) relates to probiotic bacteria, *Lactobacillus casei* and bifidobacteria, with metallic ions and/or metal NPs, to (1) use probiotics for the prophylaxis/treatment of mineral deficiency diseases; (2) use the bacteria as contrast agent for imaging of the digestive tract, and (3) use bacteria for the treatment of cancer. The authors selected a lactic acid bacterium and a bacterium of the genus *Bifidobacterium* comprising at least one metal NP bound to its surface. Metallic NPs comprised of elements like iron, manganese, cobalt, nickel, calcium, zinc, magnesium, potassium, copper, chromium, selenium, silicon, iodine, and a combination thereof (Domínguez Vera et al. 2014).

Besides the NP strategies that consist of the bioengineered bacteria, there are other strategies that developed NPs aiming to potentiate the prebiotic effect. Ha et al. (2016) developed WPI/inulin nano complexes for the delivery of resveratrol, to study how WPI and inulin concentration levels affected the physicochemical properties of nanocomplexes and to investigate the potential prebiotic effects of nanocomplexes. WPI/inulin nanocomplexes were prepared by using the modified ionic gelation method with $CaCl_2$. They demonstrated that nanocomplexes formed of inulin exhibited the potential prebiotic effect on *L. acidophilus* ATCC 43121 and the concentration of WPI and inulin were key factors that affected the physicochemical properties of WPI/inulin nanocomplexes and had a potential prebiotic effect (Ha et al. 2016).

Biodegradable NPs with lyophilized probiotic extract (filtered and lyophilized cell-free supernatant) were studied by Saadatzadeh et al. (2012). These NPs were prepared using chitosan/poly (D,L)-lactic-co-glycolic acid by double-emulsion solvent evaporation technique. Colitis was induced to male Wistar rats, and oral gavage of NPs was performed in water for 10 days. The authors observed that free probiotic extract from *L. casei* ATCC 39392 had positive effects in reduction of disease. However, the treatment using probiotic extract-loaded NPs was more efficient in mitigating the experimental colitis in comparison with the highest dose of the free probiotic extract (Saadatzadeh et al. 2012).

Feher (2012) patented a new method for preparing probiotic NPs from natural sources. The invention consists in the preparation of NPs containing probiotic extracts for medical, nutritional, or cosmetic application, for different administration routes: systemic or topical application, enteral or parenteral, and oral or intranasal administration. The applications of these probiotic NPs are for the treatment of infective diseases, traumas, age-related diseases, autoimmune diseases, inherited diseases or conatal diseases, as well as functional diseases or disorders. The use of NPs derived from killed probiotics is a novel approach to rebuild the symbiosis of host and probiotics, in the opposite way which suggests that restoring the mucosal surfaces should be by live probiotic uses. The invention described methods for in vitro preparation of NPs from probiotics mimicking as much as possible as the in vivo physiological processes (Feher 2012).

Many encapsulation techniques have been devised to protect the bacteria from adverse environmental and processing conditions and also in in vivo conditions. Commonly, the immobilization of nanocarriers on the surface of bacteria is reported.

However, there are other nanoapproaches to improve the viability of probiotics and their application (Priya et al. 2011). As mentioned above, LbL employed to form the assembly of layers on the surfaces of solids is a novel approach in the development of nanolayers or thin films to be used in drug, nutraceutical, as well as gene delivery and in biosensing (Pathak and Akhtar 2019). This technique can be exploited for the formation of nanocages on living microorganisms too (Priya et al. 2011).

Lyophilization and spray-drying have been the most studied technologies. However, the loss of bacterial viability through damaging of bacterial membranes or other cellular structures and the time-consuming effect associated are the most important disadvantages for powdered probiotics formulations. Wagner et al. (2015) suggested tablets with the formulation of dried bacteria. Tablets can provide easy administration and long-term stability and optimize the adhesion and colonization of bacteria to the epithelial mucosa. However, the high compression force that is needed to form the tablets can cause a significant loss of viability due to mechanical damage of the bacteria (Wagner et al. 2015). To overcome these limitations, the electrospinning is emerging as an attractive alternative method that enables drying of probiotics by the production of nanofibers from electrostatically driven jets of polymer solutions (Zupančič et al. 2018). Nanofibers can be used in wound dressings, as drug delivery systems, and as three-dimensional scaffolds for bone and tissue regeneration. Electrospinning has been introduced as a new method for incorporation of microbial cells into nanofibers, such as *L. acidophilus* or *Bifidobacterium* spp. (López-Rubio et al. 2009; Heunis et al. 2010; Fung et al. 2011; Nagy et al. 2014). Although electrospinning has been shown to be a promising process for probiotic incorporation, the effects of the process, solutions, and environment parameters on probiotic viability are still poorly understood. Therefore, Škrlec et al. (2019) developed nanofibers loaded with the probiotic *L. plantarum* ATCC 8014. They investigated a method to incorporate bacteria into monolithic poly(ethylene oxide) (PEO) and composite PEO/lyoprotectant (sucrose) nanofibers. PEO was chosen as it is a biocompatible, mucoadhesive, and water-soluble polymer that appears not to interfere with the bioactivity of the probiotic. The particular focus on the study was to initially determine the effects of several parameters such as those related to the environment (temperature and humidity), voltage, and solution parameters including the presence of lyoprotectant in the polymer solution as well as bacterial concentration (Škrlec et al. 2019). The authors showed that the most critical parameter for high bacterial viability after the electrospinning was the concentration of probiotic *L. plantarum* in the polymer solution. The relative humidity and voltage during fiber production did not have any vital impact on *L. plantarum* viability. An improvement in the bacterial viability and also higher survival during storage was observed, when a high concentration of lyoprotectants, namely, trehalose, was added. Nanofibers were able to release almost all the *L. plantarum* over 30 min, which is appropriate for local administration. This approach demonstrates the development of a promising local nanodelivery system based on the use of probiotic-loaded nanofibers that can provide high-loading and long shelf-life (Škrlec et al. 2019).

There are other forms to encapsulate probiotic bacteria, such as nanoBEADS and nanoemulsion. However, these strategies are poorly explored for nanoencapsulation of probiotics. NanoBEADS means nanosized bacteria-enable autonomous delivery systems, a new concept of NPs linked to the probiotic surface. Traore et al. (2014) developed nanoscale NanoBEADS comprised of flagellated *Escherichia coli* bacterium loaded with an assembly of spherical polystyrene NPs. Flagellated bacteria possess specific self-propulsion features, capable of moving through the highly viscous fluid and porous semisolid environments effectively (Traore et al. 2014; Pathak and Akhtar 2019).

Nanoemulsion is formulated using coarse emulsions by reducing the emulsion droplet dimension with high energy techniques such as high-pressure homogenization, microfluidization, and high-power ultrasound. Nanoemulsion is mostly used to load drugs and to provide effective drug delivery but also can be used to initiate the release and absorption of loaded bioactive and food agents. Nanoemulsions were designed to enhance the bioavailability of lipophilic bioactive agents in fruits and vegetables (Pathak and Akhtar 2019).

17.6 Conclusions

Nanotechnology, based on particle size, is a continuously growing field of interest in the realm of health promotion and disease prevention involving a multifaceted approach covering science, engineering, and technology. Current knowledge of the human gut microbiota and its role in gut health homeostasis (microbiota dysbiosis and chronic inflammatory diseases onset) have prompted the use of probiotics, prebiotics, synbiotics, and derived postbiotics, as efficient health-promoting strategies to maintain or recover the normal mucosal immunity and intestinal ecosystem balance. Within this preventive therapy concept, the use of commensal bacteria as probiotics is currently being explored paving the way to a new type of probiotics commonly called NGPs where *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, among others, are promising candidates. To ensure successful delivery of probiotic benefits to the consumer, several criteria are needed including high yields and stability, and solutions must be able to meet with requirements that ensure high performance and quality, including recommended dose at time of consumption. An integrated selection of production process, product formulation, and strain leads to high-quality probiotics to be included in a wide variety of delivery vectors to meet consumer needs.

Innovative approaches to guarantee optimal probiotic delivery and efficacy within the gastrointestinal tract have looked upon encapsulation strategies to entrap and protect more sensitive probiotic strains from the harsh environmental conditions encountered during production, storage, and gastrointestinal tract passage. The emergence of nanomedicine has made nanotechnology a promising tool to foster probiotic strain efficacy in gut health. Despite the constraints associated with probiotic bacteria average size not being at the nanoscale, different nanocarriers

based on size, composition, morphology, surface area, and charge provide interesting opportunities for the food sector, including NPs, nanofibers, nanoBEADS, and nanoemulsions. This large array of structural arrangements makes nanosystems quite versatile and effective.

Nevertheless, dietary applications of these nanosystems are still in its early infancy, and while the effectiveness of partnering nanotechnology with current probiotic/prebiotic/symbiotic approaches seems promising and of broad potential, additional studies are required to understand their dietary/preventive/clinical role, potential risks, and health promotion capacity.

Acknowledgments Acknowledgments to FCT/MEC (PIDDAC), due to the support with national funds through project references IF/00588/2015 and CEECIND/00520/2017 and by Operational Program Competitiveness and Internationalization in its FEDER component and by the budget of the Foundation for Science and Technology, I.P. (FCT, IP) in its OE component, project reference POCI-01-0145-FEDER-031400, with scientific collaboration of CBQF under the FCT project UID/Multi/50016/2013.

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Chapter 18
Advanced Nanomaterials in the Clinical Scenario: Virtues and Consequences

Mantosh Kumar Satapathy, R. Lekha, Samir Mehndiratta, Papita Das, and Chih-Hwa Chen

Abstract Nanotechnology is the recently developed scientific discipline that copes with the application of nanoscience with the engineering of functional systems at the molecular extent. In the past era, the nanomaterials, "the basic units of nanotechnology," were considered as the passive nanoscale particle structures or materials designed to perform only one task. Currently, nanotechnology is considered a broad and interdisciplinary area of advanced scientific research, which has a high impact on different fields of science, including biomedical research. Throughout the years of research, these nanomaterials are transformed into advanced nanomaterials or multifunctional nanoparticles (in medicine) which have different properties enabling those as multitasking nanoscale materials. For example, in the current scenario, the technologies developed for advanced nanomaterials are considered to have the potential for transfiguring how biomaterials are synthesized, functionalized, and utilized in different biomedical and medical applications such as actuators, drug delivery biomaterials, and biosensors. Even though these advanced nanomaterials

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_18

are already having a significant commercial impact in the biomedical field, which is increasing day by day, it has its pros and cons, taking into account different issues or ideas which are discussed in this chapter in detail.

Keywords Advanced nanomaterials · Biomaterials · Drug delivery · Nanotechnology

18.1 Nanomaterials: An Introduction

18.1.1 What Are Nanomaterials?

Nanomaterials contemplate the substances which have nanoscale dimensions less than 100 nm. Scientifically speaking, a nanomaterial can be of natural or artificial (incidentally formed or manufactured material) origin that contains particles in an unbounded or aggregate or whole agglomerate in which 50% or more of the particles in it has a size distribution or dimensions in the range between 1 and 100 nm (Bleeker et al. 2013). A nanometer is a scale measuring one-millionth of a millimeter that means approximately 100,000 times smaller than the diameter of a human hair. Nanomaterials are of great interest because of its minute scale measurement size along with its unique characteristics, including individually and merged properties such as optical, magnetic, and electrical. These emergent characteristic properties have a great application potential, especially in electronics, biomedical, and other scientific fields.

18.1.2 Nanoscale and Nanostructures

Nanotechnology and nanoscience are associated with materials and structural modifications that are on the nanoscale (between 1 and 100 nm). According to the Food and Drug Administration (FDA) regulations, the word "nano" is a prefix like the others that we use, for example, "milli" and "mega," and nanoscale means those that come under the nano, which is only a ten or hundred atoms in size. That is 10^{-9} which can be simplified as 1 nm = 10^{-9} m. We can generally say that there are a million nanometers (nm) in 1 mm. Compared to the nanoscale, a virus, red blood cells (RBCs), and human hair are considered approximately larger than a nanomaterial with a measurement about 10^{-6} (1 μm), 10^{-5} (1 μm), and 10^{-4} (100 μm) (Fig. 18.1).

The structural alteration of nanomaterials or nanostructures based on the morphological characteristic's modification differs significantly according to their material chemistry, composition, crystalline structures, and designing, processing, and manufacturing scheme. Recent advancements in existing synthetic methods for

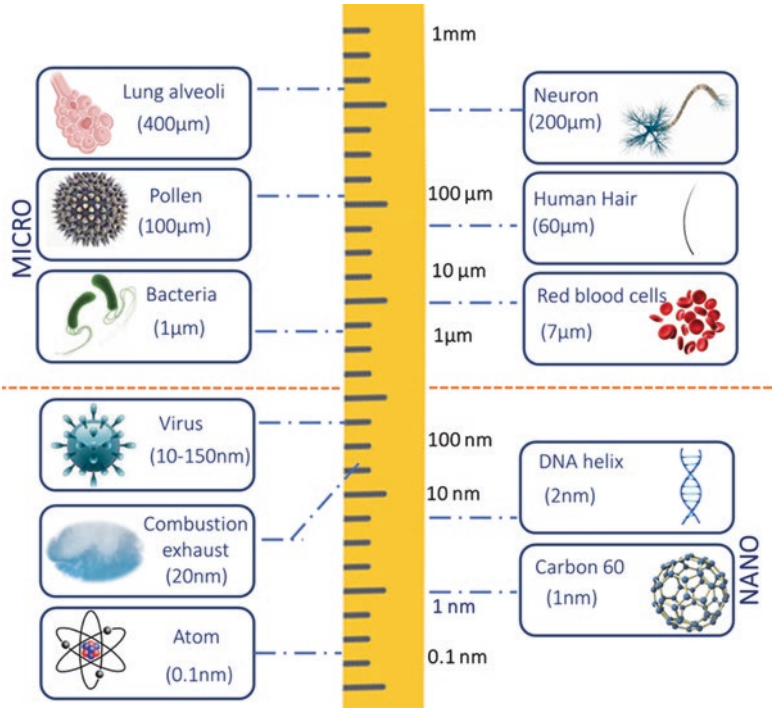

Fig. 18.1 Schematic diagram representing the size comparison of various nanomaterials (Buzea et al. 2007) (Permission under ©2019 AIP Publishing LLC and a Creative Commons Attribution 4.0 International license)

nanomaterials allow the production of various shapes of nanomaterials, including spheres, rods, tubes, needles, cubes, and octahedrons of different sizes in nanoscale. In addition to that, the morphological characteristics in relation to other chemical and physical characteristics of the nanomaterial influence each other and increase the functionality of the nanomaterial in different clinical applications. For example, studies stated that there is a remarkable relationship between the morphology of the silver nanoparticles, including its size, shape, and optical property. This characteristic feature together increases the range of its high performance in different applications. It is said that considerable effort and investment are focused on the delivery of silver nanoparticles with the precise morphologies (Fig. 18.2) (González et al. 2014)

Various studies reflect the morphological diversity of nanoscale materials if built by using infinite numbers of existing organic molecules. For example, the usage of self-assembling duplex DNA as building blocks brings about the controllable production of three-dimensional (3D) structures ranging between the sizes of 10 and 100 nm. This is one of the advanced techniques used to create nanoscale "DNA origami": polygon frameworks, gears, bridges, and bottles (Fig. 18.3) (Dietz et al. 2009; Douglas et al. 2009).

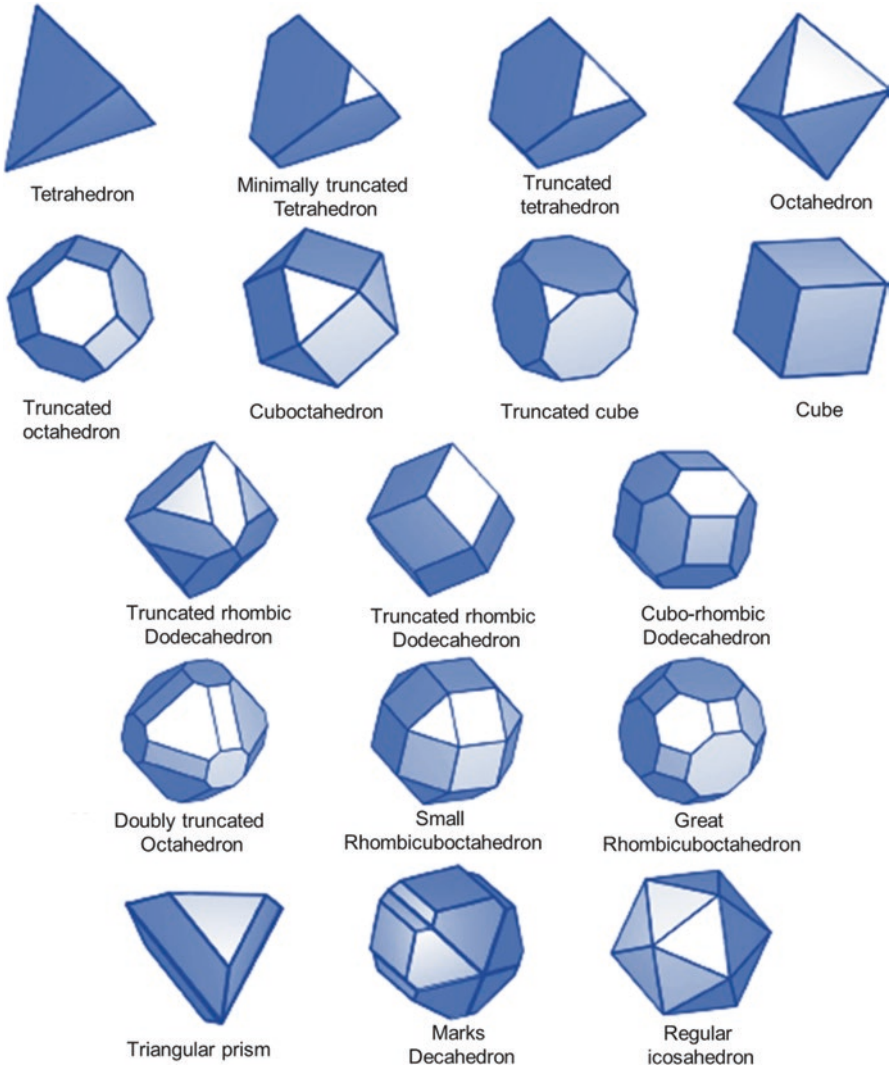

Fig. 18.2 Schematic representation of the of silver nanoparticle with different morphologies (González et al. 2014) (Reprinted with permission from American Chemical Society under Copyright © 2014)

Studies state that the morphology variation can be an effective way of controlling the functionality of nanomaterials, which can also affect their biocompatibility since it is considered as a reflection of the product of surface (interface) evolution (transformation) in the process of making of material. The diverse morphological characteristic features are of great importance for nanomaterials' quality adjustment due to the presence of many surface atoms that determine their physical as well as

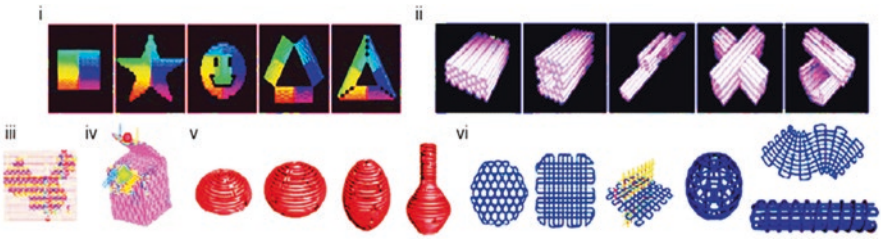

Fig. 18.3 DNA origami: (1) 2D geometry (Rothemund 2006); (2) honeycomb-lattice 3D structure (Douglas et al. 2009); (3) China map modified form (Qian et al. 2006) (Copyright Permission ©Science in China Press 2006); (4) 3D box (Andersen et al. 2009); (5) 3D structure with interconnected curvatures (Han et al. 2011); (6) DNA grating nanostructures framework (Han et al. 2013)

their chemical properties. It is a known fact that most of the nanomaterials are thermodynamically unstable with irregular morphologies (different from the shape of nanocrystals of a given substance) corresponding to the local minimums of free energy of the system.

18.1.3 History of Nanomaterials: Past to Current

It is said that the history of nanomaterials goes back to the prehistoric era, just after the big bang (theory stating the origin of the universe) since nanostructures were discovered in the early meteorites. During the evolutionary period, nanostructures are found naturally in nature like seashells and skeletons. Further, the scientific history of nanomaterials is traced much later. During the early 1857, the first scientifically reported nanoparticles are the colloidal gold particles, which were synthesized by Michael Faraday. Nanostructured catalysts have been investigated for over 70 years. However, in the USA and Germany, precipitated and fumed silica nanoparticles as substitutes for ultrafine carbon black for rubber reinforcements was discovered in the early 1940s as functional nanomaterials, followed by its commercialization. That discovery has more advancement currently as nanosized amorphous silica particles are used as a part of everyday consumer products, ranging from non-diary coffee creamer to automobile tires, optical fibers, and catalyst supports. The metallic nanopowders were developed for magnetic recording tape preparation during the 1970s. Later in 1976, nanocrystals are produced for the first time by the inert-gas evaporation technique that was published by Granqvist and Buhrman. Recent discovery includes the Maya blue paint is a nanostructured hybrid material. Even though the origin of its color and its resistance to acids and biocorrosion are still not clear, but studies of authentic samples from Jaina Island show that the material is made up of needle-shaped palygorskite (clay) crystals that form a superlattice with a period of 1.4 nm. It is said that with intercalates of amorphous silicate substrate, there are also inclusions of metal magnesium (Mg) nanoparticles due to which the blue color tone is obtained. In the current scenario, the nanomaterials have expanded

rapidly in interdisciplinary sciences ranging from experimental physics to medical applications like structural and functional materials (both inorganic and organic) with manipulative mechanical, catalytic, electric, magnetic, optical, and electronic functions (Alagarasi 2013; Ariga et al. 2016). In the current scenario, nanotechnology has reached far beyond its expectations in multidisciplinary research areas, including experimental physics, material science to medical fields, and biomedical engineering.

18.1.4 Classification of Nanomaterials

Generally, nanomaterials can be classified according to different criteria, including their source of origin, their dimensions, and their constitutive materials (Fig. 18.4). More precisely, nanomaterial classification based on their dimensions (1D, 2D, or 3D), morphology, and material composition (Fig. 18.5) is used in scientific research areas concerning their specific application in different fields. According to various dimensions, nanomaterials can be in the form of nanoparticles, nanotubes, or nanofilms. As per their compositions, nanomaterials can be made of single elements, inorganic or organic compounds, metals, or multiple elements, such as metal oxides or composites. The physicochemical properties of a material in the nanoform may vary differently according to its bulk counterpart composition. The characteristics of a nanomaterial depend not only on the type of materials but also on its size, shape, and functionalization (Buzea and Pacheco 2017).

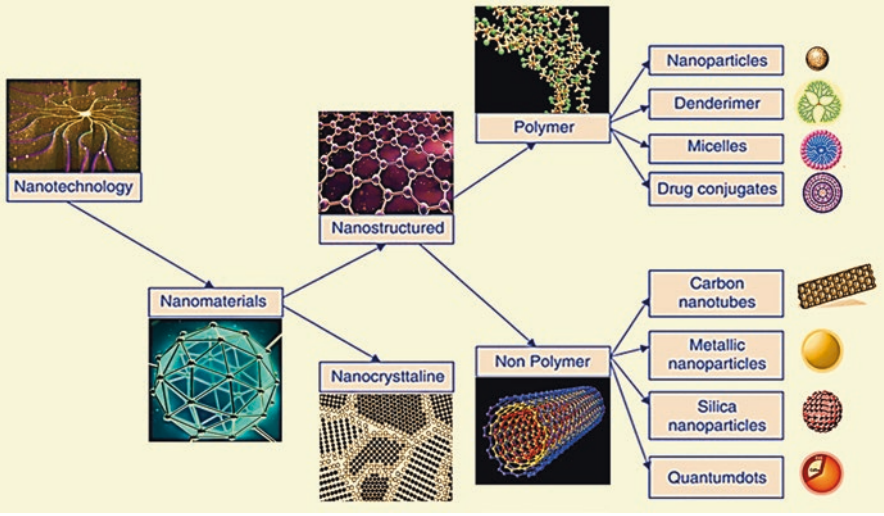

Fig. 18.4 General classification of nanomaterials

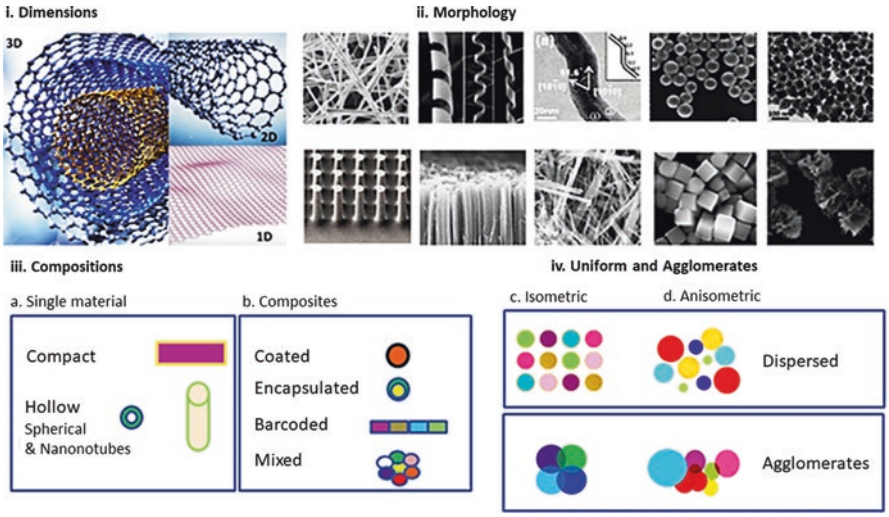

Fig. 18.5 Nanomaterial classification based on their dimensions (1), morphology (2), compositions (3), and its structural combinations (4) (Buzea and Pacheco 2017)

18.1.5 Properties of Nanomaterials

The properties of the nanomaterials include their physical and chemical properties. Nanomaterials differ from each other according to the nature of the nanomaterial, whether natural or artificial, inorganic or organic, or metallic or nonmetallic. The physical properties of the nanomaterial include the size, shape, specific surface area, aspect ratio, agglomeration or aggregation state, size distribution, surface morphology or topography, crystallinity, defect structure, and solubility which determine the ability and efficiency of the nanomaterial for its specific applications.

The chemical properties include structural formula and molecular structure, composition of the nanomaterial (including degree of purity, known impurities and additives), phase identity, and surface chemistry (composition, charge, tension, reactive sites, physical structure, photocatalytic properties, zeta potential, as well as hydrophilicity and lipophilicity) which determine the potentiality of its application in interdisciplinary research areas such as medical and industrial. Moreover, both the physical and chemical characteristic properties play an essential role in the production of a successfully engineered nanomaterial.

18.2 Advanced Nanomaterials

Advanced nanomaterials are structures at the nanoscale with specific self-assembly designs. They have unique electronic, optical, mechanical, magnetic, and catalytic properties that cannot be achieved without their nanoarchitecture. These advanced

nanomaterials provide unprecedented opportunities for tuning their properties in a vast range. It is considered as a developing field of modern research with a wide spectrum of applications ranging from nanoelectronics and energy harvesting to biology and nanomedicine. In biomedical and medical research, these advanced nanomaterials are the nanoparticles and their complexes that can be used in medicine having multiple functions. Therefore, they can act as a contrast agent in diagnostics, a biosensor, and a vector for targeted drug delivery of drugs or have a therapeutic effect for different disease conditions.

Nanosomes are the multifunctional dynamic nanoplatforms comprised of interconnected nanomodules having specific functions to be used in the field of medicine. These biomedical nanoparticles comprising a nanosome can carry drug molecules for targeted delivery, while others can fulfill the role of biosensors (pH, redox potential, membrane potential). Others may carry nanoantennae, which are made up of gold nanocrystals that heat the nanosome when placed in an electromagnetic field with a particular frequency, which enables them to be used as a diagnostic tool. Superparamagnetic nanoparticles (mainly consisting of ferrous (iron)) attached to a nanosome enable nanosome visualization using tomography, including MRI and CT scanning. Fluorescence-based nanomodules are also used in medical diagnostics to monitor the efficacy of nanomedical therapy, such as tumor cell death analysis. Depending on a specific medical objective, nanosomes of the advanced nanomaterials comprised of different functional modules can carry out several tasks, e.g., internal environment monitoring, visualization of target cells, drug delivery, and controlled drug release, treatment monitoring. Non-modular multifunctional nanoparticles also play a significant role in the medical field, for example, modified viral capsids. Nanosomes and multifunctional advanced nanomaterials or nanodevices may be considered as prototypes of medical nanorobots in the coming future (Freitas 2005).

Out of the most advanced nanoparticle used in the medical field, majority of them comprised of the polymer. A polymer is a substance that has a molecular structure built up chiefly or entirely from many similar monomer units that are bonded together in primary, secondary, or tertiary structures. In general, the multifunctional medical polymeric nanomaterial comprises of three characteristic features: the solubilizing block, an active pharmaceutical agent (pharmacon), and a targeting device (transport system).

The solubilizing block consisting of the polymer chain enables the nanoparticle to functionalize in the biological medium, including the blood and lymph. The particle's characteristics, including the hydrophilicity, hydrophobicity, electrostatic charge, and its density, will affect the pharmacokinetic and pharmacodynamic properties of the drug that is meant to be incorporated into the particle. The polymer chains may differ in terms of stability, size, composition, and the presence of specific domains (e.g., hydrophobic segments). Polymer molecular weight affects the membrane-penetrating ability of the drug in passing through the blood-brain barrier and endocytosis. The active pharmaceutical agent or pharmakon that may be bound to the polymer base or enclosed in a nanocontainer with a biodegradable or nondegradable bond can become an inactive drug precursor or an active metabolite with

the active pharmaceutical ingredient. A "targeting device" acts as a vector that can be represented by antibodies in the affected area that has a protein domain with target-specific binding affinity. The device guides the nanoparticle to a specific segment of tissue or target organ (Freitas 2005).

18.2.1 Copolymer, Block Copolymers, Nanofibers, and Nanotubes

A copolymer is considered as a polymer formed when two or more different types of monomers are linked in the same polymer chain, as opposed to a homopolymer where only one monomer is used. Copolymer sequence is another feature that can have a substantial impact on the macroscopic behavior of a polymer synthesized from more than a single monomer. Some of the possible copolymer architectures include random, alternating, block, and graft copolymerizations (Huang and Turner 2017) (Fig. 18.6).

On the other hand, when different monomer units are chemically grouped separately along the polymer chain, they are known as block copolymers. Few of the many architectures of block copolymers are linear, branched (graft and star), and cyclic molecular architectures (Fig. 18.7). Thanks to the advancement of polymer synthetic strategies and techniques, e.g., controlled polymerization techniques along with facile post-polymerization functionalization, BCPs with precisely controlled molecular weights and defined macromolecular architectures can be prepared. The extraordinary structural and compositional versatility of BCPs has facilitated an explosion in the discovery and implementation of innovative synthetic strategies capable of generating previously unattainable levels of man-made architectural complexity (Mai and Eisenberg 2012).

Fig. 18.6 Diagrammatic representation of copolymer architectures and including random, alternating, block, and graft copolymerizations (Mai and Eisenberg 2012) (Copyright license ©Royal Society of Chemistry 2019)

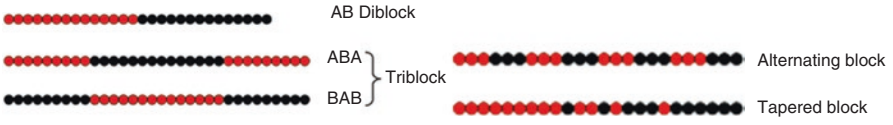

Fig. 18.7 Diagrammatic representation of block copolymer architectures including linear, branched (graft and star), and cyclic molecular architectures (Mai and Eisenberg 2012) (Copyright license ©Royal Society of Chemistry 2019)

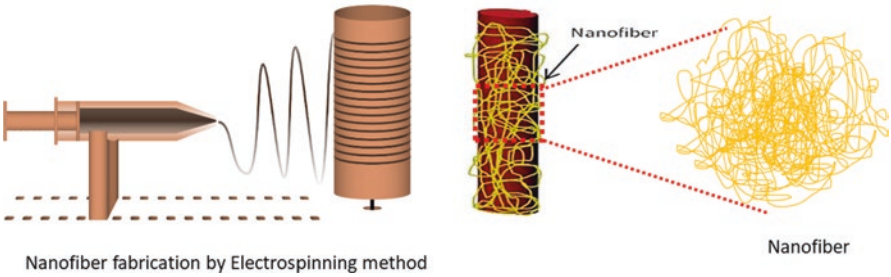

Fig. 18.8 Diagrammatic representation of nanofiber and its fabrication by electrospinning method

Nanofibers are defined as the structural fibers with diameters in the nanometer range. These filaments are prepared in micro-nanometer diameter range mostly by electrospinning (Fig. 18.8). In lab-scale electrospinning, a polymer solution is typically placed in a syringe and subjected to a strong electric field between the needle tip and a collector. If the electric field strength is enough, it will deform the pendant drop at the tip of the needle enough to eject a jet of solution which will travel toward the collector. In this meantime, the jet stretches immensely, and the solvent evaporates, leaving a porous unknitted sheet of thin polymer fibers on the collector surface. Various types of nanofibers are fabricated from different polymers resulting in diverse physicochemical properties and applicability. Due to nanorange diameters, nanofibers have a very high surface-to-volume ratio, ensuing them porous in nature for their multiple potential applications. A broad division of the application areas for nanofibers is biomedical engineering, environmental engineering, biotechnology, energy, and electronics, as well as defense and security (Ramakrishna 2005; Huang et al. 2003).

A nanotube is a tube-like nanomaterial. Nanotubes are a unique class of nanomaterials because of their larger surface areas than similar masses of bulk materials as distinctive property (Rao et al. 2001). Nanotubes are considered unique depending not only on their composition but also on their geometry (Steinhart et al. 2004). The diameter of the nanotubes, as well as the width of the walls, its length, chirality, van der Waals forces, and quality, all together affects the properties and the performance of the nanotubes (Rahmat and Hubert 2011). This characteristic feature of the dependence on geometry is what makes scaling-up nanotubes to form bulk material so challenging. Nanotubes are also considered unusual because they usually stick together in the form of bundles or strands. Nanotubes can be formed by a variety of materials including carbon and titanium, but are easier to synthesize in two-dimensional (2D) layered compounds such as graphite and hexagonal boron nitride (h-BN) (Yan et al. 2015; Wen et al. 2016; Terrones et al. 2007). While it is possible to form nanotubes three-dimensionally (3D), such nanostructures exhibited a faceted morphology and are considered as more reactive. In comparison, the preferential formation and stability of nanotubes in the case of 2D structured materials may be attributed to weak Van der Waals bonding. In the case of 3D structured nanomaterials, the presence of strong bonding in all three spatial directions makes it

difficult to achieve a stable structure. Among nanotube materials, carbon nanotubes are the most used nanomaterials.

18.2.2 Nanobiosensors: As New Members

The qualitative and quantitative detection system for specific molecules and components in diagnosing the direct or indirect physiological state of a living being (Chamorro-Garcia and Merkoçi 2016) by the composite mixture of nanomaterials with biosensors are called as "nanobiosensors" (Malik et al. 2013). Nanobiosensors as analytical devices recently have been developed enormously with ideal characteristic features such as smaller size, more portability, sensitivity, as well as specificity due to the central role of nanomaterials used for amending the sensor (Singh et al. 2020). Early-stage diagnosis of severe pathological conditions is one of the significant objectives in the advancement of nanobiosensors for the detection of specific biomarkers at low concentrations in complex sample media (Giljohann and Mirkin 2009). Biomarkers (Kumar et al. 2006) are targeting objectives in pathological conditions. Nanobiosensors can be the next-generation affinity biosensors as biomedical devices for personalized, quick-onsite diagnosis, which may overcome the hindrance of conventional biosensors.

18.2.3 Nanotoxicology

Nanotoxicology is one crucial discipline and debating topic, which includes the study of the potential adverse impacts of nanomaterials (Donaldson et al. 2004). Although nanosize as a key factor has lots of benefits in nanosize materials, they are also responsible for potential toxicity due to large surface area-to-volume ratio along with other properties (chemical composition, shape, surface structure, surface charge, aggregation, and solubility (Nel et al. 2006)) and the presence or absence of functional groups of other chemicals. Humans and animal exposure to nanomaterials happen through breathing, dermal skin contact, oral intake, and systemic circulation (Oberdörster et al. 2005) in day to day lives. There are abundant examples of nanomaterials use in healthcare scenario, e.g., zinc oxide nanoparticles regularly used nanomaterials for personal skin and hair care, sunscreens in the paint (Blinova et al. 2010; Brar et al. 2010; Dechsakulthorn et al. 2008; Fan and Lu 2005) with microbial killing (generating ROS to cause DNA cleavage, induce lipid peroxidation, and generate endogenous DNA adducts) and UV protecting ability (Wang et al. 2013). Titanium dioxide nanoparticles, widely used as food additives and drug delivery agents, another nanomaterial (Etheridge et al. 2013; Ray et al. 2009; Yin et al. 2013), cause ROS-mediated toxicity. Nano-Fe_3O_4 coated with polyethylenimine and polyethylene glycol (Hoskins et al. 2012; Wang et al. 2012), also another example, causes a severe biological issue (Mahmoudi et al. 2011). Abundantly used

silica and mesoporous silica and colloidal silica, with specific surface area and pore volume, possess various modes of cytotoxicity affecting the immune response in the living organism. Copper and modified copper nanoparticles exhibited different surface oxidation reactivity resulting in the generation of ROS at different levels (Shi et al. 2012). Nonetheless, every nanomaterial must require careful toxicopharmacological assessment based on various parameters such as its possible routes of exposure, dose metrics, and biocompatibility with expected pathological scenario associated with it (Oberdörster 2010). Therefore, nanotoxicology as a major concern should be studied critically with individual property assessment of the nanomaterials to avoid the health risks associated with exposure to existing and future nanomaterials. It would make an important role to the development of a sustainable and safe future well-being in improving healthcare. Nanotoxicology research must be focused on engineering the next generation of nanomaterial-based technologies with less possible toxic impact on human health.

18.2.4 Nanodiagnostics

Nanodiagnostics is defined as the use of nanomaterial and techniques in clinical diagnostics. Simply, when nanomaterial-based developed technique covers diagnosis and therapeutics for infectious pathological conditions in improving medical needs, it is called nanodiagnostics (Alharbi and Al-sheikh 2014). Point-of-care tests (POCTs) (Laksanasopin et al. 2015) have been improved along with nanomaterials. Recently, developed nanodiagnostics could have the benefits to overcome the associated challenges with conventional diagnostics like high cost, low portability, and potential cross-contamination giving false-negative results in the clinical diagnosis of infectious diseases (when diagnosed based on culture and microscopy, immunology, and PCR) (Wang et al. 2017). The different size of nanomaterials alike to most biological molecules boosts their usefulness for both in vivo and in vitro clinical applications (Curtis and Wilkinson 2001). In general, the basic of nanodiagnostics is binding of a nanomaterial−/nanoparticle-labeled probe to the specific target biomolecule generating a signal qualitatively or quantitatively assayed after that. Further, due to the unique surface-to-volume ratio, nanomaterials have broad advantages which can meet the requirements of clinical diagnostics with higher sensitivity (Baptista 2014) by attaching themselves to lots of targeting molecules (Tallury et al. 2010; Liu et al. 2017). Nanodiagnostic materials and devices include a variety of cantilevers, carbon nanotubes, dendrimers, nanocrystals, nanowires, nanoparticles, nanoshells, metal nanoparticles, and quantum dots which are the most used and promising nanostructures (Alharbi and Al-sheikh 2014). Despite many significant advantages over conventional diagnostic strategies, nanodiagnostics are still in the early phase of development. To make it a universal approach, more research efforts from clinical perspective are still needed for its advancement.

18.2.5 Nanocoatings and Surface Modifications

Recently nanocoating has an increasing demand in healthcare sectors. When the coating is done on the materials as thin films of nanoscale in order to modify or improve a material's functionalities such as antifouling and antibacterial properties, it is called nanocoatings (Mandracci et al. 2016). Nanocoatings offer considerable benefits for applications in medical care and medical care product industries. Moreover, multifunctional nanocoatings in products have potential applications (Mathiazhagan and Joseph 2011). Antibacterial nanocoating is a significant breakthrough applied for medical devices and implant materials and in pharmaceuticals (Arsiwala et al. 2013). For instance, nanocoating research is focused on coating the materials with antibacterials like silver and zinc nanoparticles with resulting products that can act only against the pathogens and are inert to the organism when it is intended for use externally or internally (McGuffie et al. 2016). Another research study using novel coating nanofibers releasing antibiotics when implanted in vivo during total joint replacement surgery has a potential impact in preventing severe bacterial infections (Campoccia et al. 2013). The healthcare cost can be reduced in terms aseptic condition maintenance by protecting against bacteria and other harmful contagious microorganisms if the medical and hospital equipments are coated by nanoantibacterial formulations (Vasilev et al. 2009). For example, nano antibacterial coating is done on the surfaces of medical catheters, nanoformulated antibacterial paints are used to coat operating tables, doorknobs, and door handle in hospitals and ultra-hard porous coatings are used for surgical and orthopedic implants like screws, plates, or joint implants. However, the challenges in nanocoatings for medical and healthcare sectors include biocompatibility concern; nanocoating adhesion may create toxicity, may not be uniform over the devices, and may affect the efficacy, durability, and antibacterial activity (Cloutier et al. 2015). In the future, the antimicrobial coating in the nano-formulations should be researched on a broad-spectrum range of the pathogenic microorganism without affecting the material property, stability, and mechanical and physicochemical properties of the core device in the wide range of medical applications. At the same time, the nanocoatings should minimize ecotoxicological risks and hazards (Adlhart et al. 2018).

18.2.6 In Tissue Engineering and Regenerative Medicine

In tissue engineering and regenerative medicine, the ideal scaffold design is the vital objective for its success if it can accurately mimic the architecture and composition of the extracellular matrix of the surrounding tissue, most importantly with suitable biocompatibility without toxicity. Nanotechnology also has a significant impact on nanostructure scaffold material fabrication which can induce cellular attachment, proliferation, and promoting tissue regeneration in a better way in comparison to

other scaffolds (España-Sánchez et al. 2018) due to unique physical dimension, shape, and composition of nanomaterial (Leydesdorff and Zhou 2007). Particularly, if cells are combined with nanostructured materials, it has excellent significant for tissue regeneration in vitro and in vivo (Zhang and Webster 2009). There is a broad range of nanomaterials used directly and as additive materials in regenerative and tissue engineering applications. These include crude organic materials, such as polymers, lipids, dendrimers, nanogels, nanoemulsions, supramolecular structures, and inorganic nanomaterials such as carbon nanostructures, metallic nanoparticles (such as silver, copper, gold, titanium dioxide, titania, zirconia, silver, diamonds, iron oxides), quantum dots, and magnetic nanoparticles for tissue regeneration (España-Sánchez et al. 2018). Further, the combined use of stem cells and nanoparticles has improved cell proliferation and differentiation, used in different diseases, such as ischemic stroke, spinal cord, multiple sclerosis, Parkinson, Alzheimer's, and others (Dayem et al. 2016; España-Sánchez et al. 2018). The adverse impact of many of these nanoparticles may have toxic effects on cells (Ai et al. 2011). The physical and chemical properties of the nanomaterials should be studied carefully before their tissue engineering applications. Future nanomaterial advancement from tissue engineering and regenerative medicine viewpoint comprises of adequate biocompatibility and targeted specificity in control manner with less possible evidence of toxicity (Fathi-Achachelouei et al. 2019).

18.2.7 In Drug Delivery and Radiotherapy

Nanomaterials for drug delivery and radiotherapy mainly focus on improved cancer diagnosis treatment with novel responsive nanoparticle systems subjected to tumor microenvironment targeting and cancer therapies (Cheng et al. 2012; Prasad et al. 2017). Currently, various nanomaterial-mediated drug delivery systems such as liposomes, nanoparticles (NPs), polymersomes, dendrimers, nanotubes, and hydrogels are promising strategies to enhance drug delivery efficiency as well as therapeutics (Bamrungsap et al. 2012; Liu et al. 2007; Jabir et al. 2012). Radiotherapy is a specific therapeutic approach mainly used to treat various types and grades of cancer (Baskar et al. 2012; Miller et al. 1981; Sheline 1977). Targeted radiotherapy for cancer by specific nanomaterials such as pure carbon-based particles, fullerenes and nanotubes, various organic dendrimers, liposomes, and other polymeric compound nanomaterials is ever exciting. These vehicles can distinctively interact with cell-surface tumor antigens to deliver radioisotopes to the cancer cells. In general, radio-based nanoparticle systems are unique due to their activation by various energy sources, such as light, magnetic field, radiofrequency, microwave, and ionizing radiation, which make them attractive and efficient antitumor vehicles for cancer treatment. Radiotherapy is improved by using high-atomic number NPs (e.g., gold, gadolinium, platinum, iron oxide, hafnium) due to potential release copious electrons, ion, and photons thereby amplifying radiation-induced cancer cell targeting which offers its promising future and clinical translation (Bergs et al. 2015;

Kuncic and Lacombe 2018; Ma et al. 2017). More interestingly, radiotherapy, along with nanoparticles, is attributed to better drug delivery due to their synergistic interaction inducing different modes of cancer cell killing. This phenomenon must be studied carefully by current research specifically on the mechanism of action and the interaction between nanoparticles and radiation during radiotherapy (Spyratou et al. 2017; Townley et al. 2012).

18.2.8 In Surgery and Visualization Techniques

Current drug delivery systems, gene therapies, body and organ imaging, surgical tools, and diagnostic procedures necessitate nanomaterials, nanomedicine, and nanotechnology and their advancement for highly specific molecular interventions in disease diagnosis and treatment (Petersen et al. 2014). During surgical interventions following visualization techniques for dreadful pathological conditions, nanotechnology and nanomaterials have tremendous contributions reducing the pain, morbidity for target-specific therapeutics, and its advancement (Abeer 2012). Research on nanorobotics and nanomaterials together for the development of minute surgical instruments is a recent fascinating trend that can be used to perform diagnosis, microsurgeries, and drug delivery at the complex and sensitive organs like eyes, brain, lungs, mucous membranes, and minute vessels (Bar-Cohen 2005). Further, surgery guided by visualization aid can also improve the therapeutics. For example, "nanocameras" can provide close-up visualization of the surgery in tissue, cell, and genetic levels. Nanomaterial-based approach can also explore the causes of relatively new diseases for its future prevention (Abeer 2012). In this context, nowadays gold nanoparticles offer lots of advantages over other nanomaterials because its stability, ease of synthesis, cell internalization, and excellent biocompatibility (Jiang et al. 2008; Toy et al. 2014) promote the radiation effects (Chithrani et al. 2010; Bhattarai et al. 2017) at cell site. Other theranostic nanomaterials (such as gold and gadolinium) have also been developed with higher treatment efficacy together with target-specific diagnosis ability (Luchette et al. 2014) in comparison to only radiation therapy.

During visualization techniques, nanoparticles, including quantum dots, fluorescent semiconductor nanocrystals, and magnetic nanoparticles, have proven their excellent properties for in vivo imaging techniques in several modalities such as magnetic resonance imaging and fluorescence-based imaging (Ryvolova et al. 2012). Magnetic nanoparticles such as paramagnetic, ferromagnetic, and superparamagnetic particles make these materials useful for magnetic drug targeting (Arias et al. 2012), cell tracking (Banerjee et al. 2010), hyperthermia, and medical imaging (Chomoucka et al. 2010). For example, iron oxide nanoparticles coated with polyethylene glycol (PEG)-poly aspartic acid block copolymer for pancreatic cancer imaging (Kumagai et al. 2009), PEG-coated iron oxide nanoparticles as MRI contrast agents for breast cancer, lymph node metastasis detection, organic fluorophores, and ferric oxide nanoparticles with chitosan for

brain glioma diagnosis (Jiang et al. 2012) have been studied extensively for their applications in clinic. Apart from those, nano-quantum dots (QDs) (CdSe/ZnS− QDs− 2 nm-blue emission, 7 nm-red light emission (Drbohlavova et al. 2009; Yu et al. 2003)) play a significant role in detection of various types of cancers and other pathological conditions when injected in vivo in a target-specific manner. However, several toxicological researches should be performed by using these nanomaterial formulations before their use in clinics. Nonetheless, the potentiality of the nanomaterial-mediated in vivo imaging/visualization has a promising future in cancer and other therapeutics with not only better diagnosis but also improved treatment approach.

18.2.9 In Health and Medicine: Clinical Scenario

Recently, advanced nanomaterials diversely impact human healthcare. There are widely used nanoparticles and nanoformulations such as liposomes, polymers, micelles, nanocrystals, metals/metal oxides and other inorganic materials, and proteins in the recent PharmaMarket. Their classification is very important based on their approval status and under investigational status which is represented as the pie chart showing the percentage or proportionality of type of nanoparticles. Advanced nanomaterials have possible benefits like improved disease diagnosis, better drug delivery, active tissue regeneration, and other tissue engineering applications in a useful way. At the same time, the use of manufactured nanomaterials put up solemn questions regarding potential risks to human health and the environment during their daily exposure (Warheit et al. 2008). The noteworthy concern is about the potential health and environmental risks leading to alarming concerns as nanotoxicology. Nanotoxicology is the scientific discipline, including the detailed study of adverse health effects of nanoparticles (Krug 2014; Oberdörster 2010). This branch deals with the detailed study and applications of toxicity of advanced nanomaterial applications in tissue engineering, biomaterials, biosensors, and bioimaging (Nalwa 2014) to avoid future challenges and associated global health issue (Saifi et al. 2018). The associated toxicological risk to human health during inhalation and dermal exposures of nanomaterials are represented (Fig. 18.9). The detailed investigational approach is helpful for nanomaterials and nano-enabled products before their likelihood of exposure (occupational and environmental exposure), which can avoid potential impacts and risks during their future applications.

Most importantly, the FDA regulates the characteristics of the nanomaterials to be used clinically based on its safety and efficacy for its use for human, which are mostly biological products. The nanomaterial drug products must go through several phases of clinical trials before their clinical applications after FDA approval.

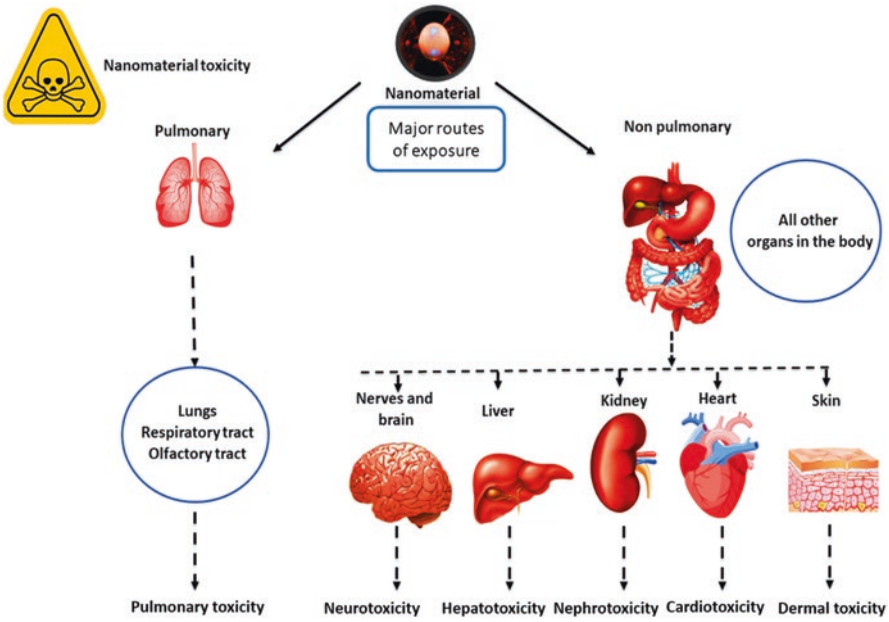

Fig. 18.9 Diagrammatic representation of nanotoxicity (affecting major organs of body)

18.3 Virtues, Consequences, and Limitations

Nanotechnology is now used in multidisciplinary areas, including chemistry, physics, biology, and advance engineering (Bhushan 2017). Advanced nanomaterials show great potential to address the clinical needs in significant medical conditions. The tininess of the particles confers useful functional properties, which make them vital to be used in different fields (Loeve 2018), such as for the treatment applications, diagnostic tools, and therapeutic agents. Recently, in biomedical and medical applications, nanomaterial (Chen and Chatterjee 2013) essentiality is very high. Studies already verified that the site and the duration of treatment induced biochemical interaction of nanomaterials, all affected exclusively by the particle size (Mu et al. 2014). Efficient drug delivery using nanomaterial is already an established field in medical therapeutics. Biological microelectromechanical devices or bioMEMS implanted into the body that can deliver doses of drugs or carry new cells to damaged tissues bring the concept of nanosurgery in the current medical research (Grayson et al. 2004). The use of nanoparticles as image enhancers is being developed as part of biomedical imaging and diagnostics (Janib et al. 2010; Lee et al. 2012). The imaging probes and the implant coatings can be inserted into the human body with particle sizes ranging from 2 to 10 nm. Studies conducted have already proved that enhanced magnetic properties of iron (III) oxide nanoparticles are

suitable for the use of contrast agents in magnetic resonance imaging (MRI). In the medical studies and treatments of cancers, advanced nanomaterials are used as carriers for delivering imaging agents to cancer cells, thus making it easier to locate the cancer cells precisely and making treatment much more effective (Cormode et al. 2009). For example, one technique being tried is to inject the patient with certain nanoparticles, often gold nanoparticles to locate the cancer cells that can be later irradiated and destroyed by different treatment methods. Hence, the essentiality and virtues of nanomaterials in specific fields, especially medical, is an inevitable area of interest.

The engineered nanostructures and particles which have been studied and proved efficacious in imaging, diagnosis, and treatment of various diseases have many advantages. Since the nanomaterial applications are being introduced increasingly in the medical industry, medicine, and pharmacology, elucidation of potential adverse health effects of nanomaterials is also necessary. However, the consequences also include toxicity and ethical issues. Studies in recent years suggest the genotoxic and carcinogenic outcomes of engineered nanoparticles. The chemistry, solubility, degree of agglomeration, size, shape, and charge of particles may influence the toxic properties of the nanoparticles. In total, the nanomaterials, when exposed to a biological system, can produce oxidative stress, inflammation, immunotoxicity, genotoxicity, and carcinogenicity. Before the commercialization of nanomedicine products, they must have to undergo extensive preclinical and clinical testing.

Advanced nanomaterials have superior functionalities. However, several limitations prohibit them from being used universally for their full-scale applications. The most critical issues by nanomaterials are the toxic impact on the environment, which further leads directly and indirectly as the potential cause of toxicity to human health as an increasing concern of today.

18.4 Conclusion

Despite the growing importance of advanced nanomaterials in healthcare as the current fascinating trend, several associated unusual pathological abnormalities come up these days worldwide. Advanced nanomaterial research as a critical approach should include the detailed analytical approach of nanotoxicology for assessing the microstructure and chemical composition of nanoscale materials. Further, the potential impacts of nanomaterials will substantially be checked based on essential key factors, whether these affect critically the environment and health followed by FDA regulations. More specifically, associated toxicity should further be checked carefully before their application in translational medicine. This necessitates several implementations and regulatory actions such as modification and control and safe use of nanomaterials based on the previous limitations of production systems. Subsequently, the realistic approach of future nanotechnology is in minimizing the nanotoxicity and development of sustainable nanomaterials with improved efficacy.

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Chapter 19
Nanomedicine: Challenges and Future Perspectives

Anju Surendranath and Mohanan Parayanthala Valappil

Abstract Nanotechnology has generated an exponentially impressive surge globally among researchers in search of inventions satisfying various needs of biomedical and healthcare modalities. As a result, rapid progress has become evident in targeted drug delivery, sustainable drug release, imaging-based therapies and related theranostics. Last few decades occupy the golden age of nanotechnology with the innovative development and fabrication of various engineered nanoparticles suitable for potent applications in biomedicine. Herein reported are the various nanoparticles that have found profound applications in the field of biomedicine and the possible applications proved to be effectively equipped in biomedical and healthcare scenario. Briefly discussed are the possible adverse effects elicited by nanoparticle exposure in the living system. Also focused on the global status of nanomedicine and concluded with upcoming strategies in nanotechnology and future perspectives.

Keywords Bioimaging · Biomedical · Drug delivery · Drug release · Nanomedicine · Nanotechnology · Healthcare

19.1 Introduction

The advent of nanotechnology, being the most revolutionized outcome of the present era, has gained spectacular status across various scientific and engineering disciplines owing to its unique physico-chemical characteristics. The concept of nanotechnology has first been envisioned by the famous physicist Dr. Richard Feynman, during a lecture delivered to the American Physical Society in 1959 said 'There's Plenty of Room at the Bottom'. A more precise description of nanotechnology could therefore be the manipulation of any matter with at least one dimension

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1_19

in the nanoscale range of 1–100 nm which intriguingly encompasses them with distinct physico-chemical properties compared to their bulk counterpart (Powers et al. 2007). Nano-structured materials can be designed so as to exhibit very specific and desired qualities at atomic precision to control their physical and chemical properties. This indirectly will benefit them with enormous qualities suitable for various application level strategies. It should be worth appreciating that nanotechnology itself is not a single merging scientific discipline rather a meeting of traditional and modern scientific concepts that offers various practical applications. Even though a vast arena of possibilities ensure with the advent of nanotechnology, a promising revolutionary advancement was found with bio-nanotechnology that offers great achievements in biomedicine. This has offered a strong foundation lending for personalized and quantitative medical care in nearby future along with improvement in diagnostic therapies.

19.1.1 Nanotechnology in Medicine

Ascribed to their exceptionally unique fundamental properties like ultra-small nanoscale structure, increased surface area, good carrier mobility, tunable band gap, potent in vivo biocompatibility and excellent in vivo biodegradability, nanoparticles (NPs) are proved to be successful candidates for various biomedical applications including drug-gene delivery, fluorescent labeling of target bioanalytes, biosensors, tissue engineering scaffolds, regenerative medicine, personalized medicine, MRI contrast enhancement and so on (Salata and Oleg 2004; Prasad et al. 2016). The latest one is nanorobotics in medicine and healthcare (Fig. 19.1).

Nanorobotics, although having many applications in various other interdisciplinary areas, has now been the most equipped technology in biomedicine for targeted drug delivery, for complicated surgeries as well as for precise monitoring and diagnosis of various disease conditions (Cavalcanti et al. 2007). Minute surgical instruments can be made out of NPs which can be used to perform microsurgeries at any part of the body with utmost accuracy (Sanchez and Pumera 2009). Precise and accurate targeting of various minute and complicated organs can be done by specifically targeting the exact site instead of damaging other organs in its immediate vicinity environment. Nanocameras have now been an innovative field of research for close-up visualization of surgery (Thrall 2004). They can provide with exact imaging of tissues, cellular as well as genetic levels during surgery. With relatively enhanced hardness and rigidity compared to their bulk part, it has been proved that NPs are attractive candidates for the fabrication of surgical implants. While considering these chemical compositions for biomedical applications, proper surface modification plays a key role to effectively stabilize the particle in the physiological system and thereby provide efficient biocompatibility.

Biocompatibility and in vivo biodegradability are therefore the two major key points to consider while selecting a nanoparticle (NP) for the desired biomedical application. The possible safety concerns raising today can probably be

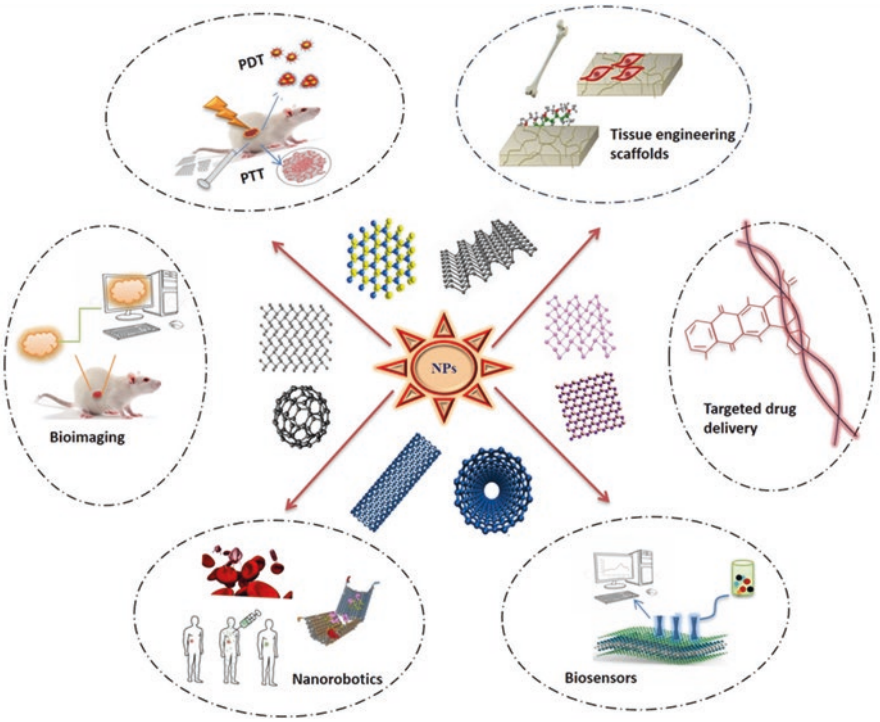

Fig. 19.1 Various applications of nanoparticles in medicine and healthcare

alleviated by this way. With nanoscale structural engineering, it is now possible to design and manipulate matter bottom-up with a precision of even less than one thousand thickness of human hair to life-saving devices to treat vulnerable diseases of mankind. A number of nanotechnology-based diagnostic aids, imaging tools and nanoscale medical instruments are already well established and are available satisfying the benchtop-to-bedside approach in medicine. With nanotechnological advancements, the new-generation lab-on-a-chip with nanoscale componentry will offer advantages including reduced costs, portability, reliability and faster analysis.

19.1.2 Generally Employed Nanoparticles in Biomedicine

The very first generation of nanoparticle-based theranostics involved lipid systems like lipid micelles and liposomes which are now FDA approved. On to these nanoformulations, later on, various inorganic NPs like gold or magnetic NPs have been clustered to form conjugates giving emphasis on drug delivery, imaging and therapeutic applications (Park et al. 2006). NP-mediated drug delivery thus reportedly

aids in preventing drugs from being blemished or degraded in the gastrointestinal tract and also helps in targeting water insoluble drugs to the desired site of action. Absorptive endocytosis is the typical uptake mechanism exhibited by NPs which helps in high degree of oral bioavailability. Generally NPs used for targeted delivery of drugs and nucleic acids in biomedicine include metallic, polymeric, organic and inorganic NPs as well as dendrimers, micelles and liposomes. A poorly soluble drug even though having potent therapeutic efficacy which often gets outdated from practical applicability is another curse in the medical sector. This can be achieved by tagging those drugs with hydrophilic NPs and delivered on to the site of action (Haba et al. 2007; Shi et al. 2008).

Polymeric NPs of size range from 10 to 100 nm are effective drug-gene carriers that are well suited for biomedical applications due to high biocompatibility and biodegradability, and the commonly used polymeric nanostructures include synthetic polymers such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), polylactic acid (PLA) and polylactic-*co*-glycolic acid (PLGA) (Zhang et al. 2008; Ensign et al. 2012; Prasad et al. 2017). Also natural polymers such as alginate and chitosan are extensively used in the fabrication of nano-formulations (Mitra et al. 2001; Watkins et al. 2015). Allotropes like graphene, graphene oxide and fullerene have recently been widely investigated as effective drug delivery systems (Montellano et al. 2011; Liu et al. 2013). Gold nanoparticles also offer unique features for these applications. Likewise various potential NPs have been extensively studied in the field of biomedicine.

19.1.3 Carbon Allotropes Like Graphene, Graphene Oxide and Fullerene

Graphene, reputed as the strongest material to date, is a carbon allotrope having an sp^2-hybridized carbon framework. With a 2D honeycomb structure of one carbon thickness, graphene NPs provide distinctive optical, thermal, electronic and mechanical properties. This can be manufactured into sheets, flakes and nanodots to provide a wide variety of applications in the field of biomedicine. Graphene derivatives such as pristine graphene, graphene oxide and reduced graphene oxide have been extensively studied for various biomedical applications (Syama and Mohanan 2019). Graphene oxide (GO) can be used to deliver anticancer drugs which are insoluble in water. Various surface modifications using PEG-like polymeric moieties enable stable colloidal dispersions of graphene. It was reported that water-insoluble anticancer drug SN38 was successfully loaded onto amine-terminated PEG-grafted GO by noncovalent adsorption and was targeted against cancer cells, which was proved to be effective in tumor therapy (Liu et al. 2008). In a very recent study, de Sousa et al. (2018) evidenced the effect of folic acid-conjugated graphene oxide for the delivery of chemotherapeutic drug camptothecin. For the delivery of genes, molecular beacons and aptamers, graphene and its derivatives are proved to

be excellent candidates by specifically detecting the biomolecules. It was reported that siRNA-mediated gene silencing with the aid of polyethyleneimine-grafted graphene oxide was successfully done which opens up a new window in gene therapy (Zhang et al. 2011). Graphene derivatives are also evidenced as good photothermal and photodynamic therapy agents in combination with anticancer drugs to treat malignant tumors. Likewise fullerenes are another class of carbon allotropes having a buckyball structure which was discovered in 1985 by laser-induced graphitic ablation, possessing distinct properties suitable for various biomedical applications. Among the various forms of fullerene, C60 and C70 fullerene NPs have attracted the modern scientific attention with its unique physico-chemical characteristics well tunable for biomedicalapplications.

Buckminster fullerene (C60), the most abundant and extensively studied allotrope, has typically a truncated icosahedron structure with 60 carbon atoms possessing C_5–C_5 single bonds forming pentagonal structure and C_5–C_6 double bonds forming hexagons (Kroto et al. 1985). Fullerenes show extreme durability and stability. Functionalized fullerenes have gained acceptance in biomedicine as they possess good stability and dispersity in the physiological system. These are experimented as potent candidates for various biomedical applications like drug-gene delivery, tissue engineering scaffolds, 3D printing applications, imaging-guided therapies and so on. Recent researches were focused on anticancer and anti-HIV treatment modalities with the use of various fullerene moieties (Marchesan et al. 2005; Krishna et al. 2010). All these carbon allotropes were proven as excellent photothermal, photodynamic as well as photo-acoustic agents which enable them for the treatment of malignant tumors. For biosensing as well as for bioimaging applications also, they showed potent ability which can be well attributed for various applications in biomedicine.

19.1.4 Metallic and Metal Oxide Nanoparticles

In recent years, a great deal of interest from scientists and engineers of nearly all disciplines has focused on metallic nanoparticles. Due to its unique physico-chemical properties such as optical, electrical, magnetic as well as specific heat and surface reactivity, it makes them suitable for various biomedical applications such as imaging, sensing, drug-gene delivery, tissue engineering and therapy. Metallic NPs are suitable markers for the optical detection of various target biomolecule analytes for antiplatelet action, antimicrobial activity, protein stabilization as well as for various imaging-guided therapies (Kim et al. 2007; Shrivastava et al. 2009). Silver NPs are one among the most accepted metallic NPs suggestive to be excellent anticoagulant and antithrombolytic agents (Deb et al. 2012). Metal and metal oxide NPs are able to distinguish bacterial cell from mammalian cells thereby preventing bacterial biofilm for a long time (Gold et al. 2018).

Antimicrobial properties of metallic NPs like silver NPs are well known. Similar to antibiotics, metal NPs are able to penetrate deeper into the bacterial cell through

bacterial metal transport systems and metalloproteins (Prasad et al. 2020). Silver, gold, gallium, platinum, zinc, magnesium, titanium-like metals and their oxides are proven to be excellent antibacterial agents. Biomedical applications of metallic NPs are a peer reviewed session in NP research. Gold NPs are excellent source for various applications in biomedicine, especially for imaging-based therapies, biosensing, drug-gene delivery, photothermal therapy as well as photodynamic therapy. But the main criterion should be taken into consideration before using metallic NPs for biomedical applications if that it must be nontoxic and biocompatible with the physiological system. Also they must be selectively addressed to target the specific site of action; otherwise accumulation can happen at undesired sites. For that, surface functionalization using biomolecules can be a suggestive way. Peptides, antibodies, aptamer and micro-RNA-like moieties are excellent agents for this purpose (Efremov et al. 2007). Metallic NPs like gold NPs and silver NPs are having great interest in biomedical scenario, especially in the form of nanorods, nanotubes and nanosheets for drug delivery, sensing and photothermal therapy.

19.1.5 Transition Metal Dichalcogenide Nanoparticles (TMDCs)

Beyond graphene, there occurred a steep hike in search of a substitute for graphene overcoming most of its defective sides, thus paving the way for search of other 2D materials. Transition metal dichalcogenide is one among the stringent discoveries of next-generation nanomaterials intended to surpass the defects of graphene and graphene-like molecules. TMDCs are materials of the form MX_2, where 'M' represents a transition metal atom from groups 4 to 10 in the periodic table (Ti, Zr, Hf, V, Nb, Ta, Mo, W and so on), and X stands for chalcogen elements (S, Se or Te). Most of all the materials of this family are semiconducting and are therefore well suited for electronic and optical applications. The potential uses of TMDC NPs for biosensing, drug delivery, multimodal imaging, tissue engineering as well as imaging-guided therapies are being actively researched nowadays due to their fascinating physicochemical properties. TMDCs have been applied for potential applications in photothermal therapy along with multimodal imaging-guided therapies for the treatment of dreadful diseases like cancer. Indeed a wide range of different TMDC-based devices have already been successfully demonstrated such as field effect and thin film transistors, photodetectors and photovoltaics. MoS_2, WS_2, $MoSe_2$ and WSe_2 are some of the TMDCs which have immense biomedical applications. Multimodality as an imaging and sensing probe is attributed due to its relatively high molecular weight and easiness of getting dopped with paramagnetic particles which imparts to their efficacy as contrasting agents for computed tomography (CT), magnetic resonance imaging (MRI) and photoacoustic imaging (PA) techniques (Yang et al. 2018).

Metallic edges and atomic defects are another key feature of 2D TMDC NPs which makes them even better candidates for antimicrobial techniques in a better way than the traditionally accepted silver NPs (Liu et al. 2017). Photothermal

therapy along with drug delivery and MRI contrast makes TMDCs an exciting material for combinatorial therapy of cancer. One recent study reported this combinatorial therapy in BALB/C mice bearing MDA/MB tumor mice (Wang et al. 2017), and similar other studies reported this efficacy of various TMDC NPs in different tumor models (Liu et al. 2014; Yong et al. 2014). Biochemical sensors can be made out of these TMDC NPs, and one such example is reported by Wang et al. (2012) for the detection of H_2O_2 with a precision limit of 0.26 μm. These are proven to be good NIR-absorbing agents as well as singlet oxygen generation species which can be employed for various diagnostic therapies apart from its commonly employed biomedical applications like drug-gene delivery and tissue engineering scaffolds. All these stringent features make them a multifunctional theranostic platform for simultaneous imaging-guided diagnosis and therapy.

19.1.6 Magnetic Nanoparticles

Magnetic NPs are promising materials for various biomedical applications. These are more widely used in drug delivery, tissue repair, cellular labeling of bioanalytes, hyperthermia treatment of cancer as well as for contrast imaging technologies in biomedicine. Materials with good enough magnetic saturation such as transition metals like Fe, Co and Ni or metal oxides like Fe_3O_4 and Fe_2O_3 are widely used. Even though pure metals possess high-degree magnetic saturation, they are highly toxic to the physiological environment and extremely sensitive to oxidation (Xie et al. 2010). Therefore without proper surface functionalization, magnetic NPs are not suitable for biomedical applications. For example, PEG-, PVA- and dextran-functionalized Fe_3O_4 and Fe_2O_3 NPs are promising candidates due to their relative ease of functionalization and high degree of biocompatibility. One among the most desirable applications of magnetic NPs is their drug loading efficiency to fight cancer, and various studies have reported that PMMA- or PEG-functionalized magnetic iron oxide NPs are efficient in loading drugs like doxorubicin, carboplatin, epirubicin, paclitaxel and 5-fluorouracil by being externally directed to focus at tumor sites. Also for imaging-guided therapies and photothermal therapy, by inducing hyperthermia, these kinds of NPs have widely been used with size ranging from 10 to 100 nm.

In cellular therapeutic applications like tissue repair, magnetic NPs are loaded on to cells and are directed to focus externally by a magnetic field to the desired sites for tissue repair. To test this strategy, several cell types such as natural killer cells, mesenchymal stem cells, cancerous cell lines and blood erythrocytes are employed. One such study was reported which uses magnetically labeled natural killer cells directed by a magnetic field towards human osteosarcoma cells in vitro to treat bone cancer (Nakashima et al. 2005). Despite cancer treatment modalities and various cell therapies, the use of iron oxide nanoparticles in bone regeneration remains largely unexplored even though it plays crucial role in bone mineralization. Pareta et al. (2008) for the first time used magnetic nanoparticles in an effort to reverse

osteoporosis. Likewise several studies were done to evaluate the potential of magnetic NPs for various biomedical applications and to prove that they possess excellent characteristics to make them flexible for various applications in biomedicine.

19.1.7 Polymeric Nanoparticles

Polymeric NPs have revolutionized biomedical technology and related fields as biomaterials for healthcare applications. They are macromolecules consisting of covalently bonded repeating monomeric subunits making them amorphous or crystalline with chains being linear, branched or cross-linked. Natural polymers such as chitosan, albumin, and heparin have been used for the delivery of oligonucleotides, DNA, protein as well as various drugs. An albumin-paclitaxel nanoconjugate has been studied in the treatment of metastatic breast cancer during phase III clinical trials (Miele et al. 2009). Synthetic polymers include polyesters, polyanhydrides, polysaccharides, polycarbonates, polyacetals and polyphosphazenes which possess a wide range of applications ranging from tissue engineering scaffolds, sustainable drug release, delivery agents and regenerative medicine to the development of life-saving devices, implants and dental products. Recent trend in polymeric medical technology has adapted a tendency to substitute degradable polymers instead of nondegradable polymers for the advancement of various healthcare modalities. They have got considerable attention for their potential in various interdisciplinary arenas which include the development of surgical suture materials, bone fixatives, vascular grafts, stents, catheters, adhesion prevention devices, artificial skin and sustainable drug delivery systems. Various polymeric NP-based products are now FDA approved for being used in the biomedical sector. One such example is the widely used resorbable suture material Dexon® (American Cyanamid Co.) which is made up of polyglycolic acid (PGA) multifilament.

Gliadel is another example of a polymeric nanocomposite used as a delivery matrix for controlled delivery of the chemotherapeutic drug carmustine to treat brain cancer, which is also FDA approved. Polyzene-F® (poly[bis(trifluoroethoxy)phosphazene], CeloNova BioSciences) is a commercially available polymeric product used for stent coatings and was FDA approved in 2008 (Radeleff et al. 2008). Also polymeric NPs are used as surface functionalizing agents for various other metallic NPs for effective dispersion and reduction of accumulation toxicity enabling easy excretion. Recently, degradable polymeric nanocomposites have risen up with stringent characteristics. Various types of degradable polymers have been developed to date having typical features applicable for various aspects in modern science. Thus being the most renovative field of biomedicine and biomedical technology, degradable polymers have gained substantial acceptance and appreciation in recent times. Current researches are focusing on the development of liquid polymer-based injectable systems which can be delivered via injection or in the form of injectable particulates that will open the gate for future healthcare applications.

19.1.8 Quantum Dots

Photoluminescent semiconductor quantum dots (QDs) having size in the range of 1–10 nm have got great attention and scientific interest from the past few decades mainly due to their unique optical and electrical properties which can be well tuned for various biomedical applications. Stringent characteristics like size-tunable light emission, improved signal enhancement, resistance against photobleaching and size-tunable multicolor emission spectra which are in contrast to organic dyes make them an excellent choice in biomedical technology. The emission wavelength range of QDs is mostly dependent on their size and chemical composition which makes them categorize into UV, visible and NIR emission QDs. QDs with NIR absorbance spectra were used for photothermal and photodynamic therapies due to hyperthermia induction possibility and ROS generation capacity. Cadmium-based quantum dots are widely studied from the past few years, but due to their inherent toxicity nowadays, various other cadmium-free carbon-based nanodots, metallic QDs and TMDC-based QDs are gaining popularity. Compared to their bulk counterpart, QDs exhibit novel optical properties due to effective quantum confinement effect of excitons and photons in nanostructures (Dutta et al. 2002).

In biomedicine, QDs are mainly used for imaging-based therapeutics, photothermal therapy and photodynamic therapy apart from usual applications like NP-based drug-gene delivery. Due to strong spectral characteristics, they can be used as contrast agents for CT, PA and MRI imaging also. Chemical moieties like chitosan, dextran, PEG and BSA and biomolecules like aptamer, peptide, siRNA and oligonucleotide can be used for effective functionalization of QDs. New QD-based devices like biosensors for advanced diagnostics, in vivo imaging, targeted drug delivery and imaging-based therapies are expected to be developed in the nearby future. Concerns associated with QDs must be systematically addressed before using them for practical commercial medical applications, and this could be achieved in the coming decade most probably.

19.2 Applications of Nanoparticles in Medicine

19.2.1 Disease Diagnosis and Drug Delivery

Recent advances in the field of nanotechnology have created an equally impressive surge in developing NP formulations suitable for various diagnostic and therapeutic applications. Majority of the NP formulations that are clinically used belong to therapeutic and diagnostic purposes. These are aimed at a more efficient delivery of chemotherapeutic drug to the site of infection and avoiding tissue/organ accumulation based on enhanced permeability and retention effect (EPR effect). Generally for drug delivery, drugs have been coated over the NP surface in the case of QDs and sheet-like 2D materials, whereas in nanotubes like TiO_2 nanotubes, drug molecules can be loaded inside for sustainable drug release (Fig. 19.2).

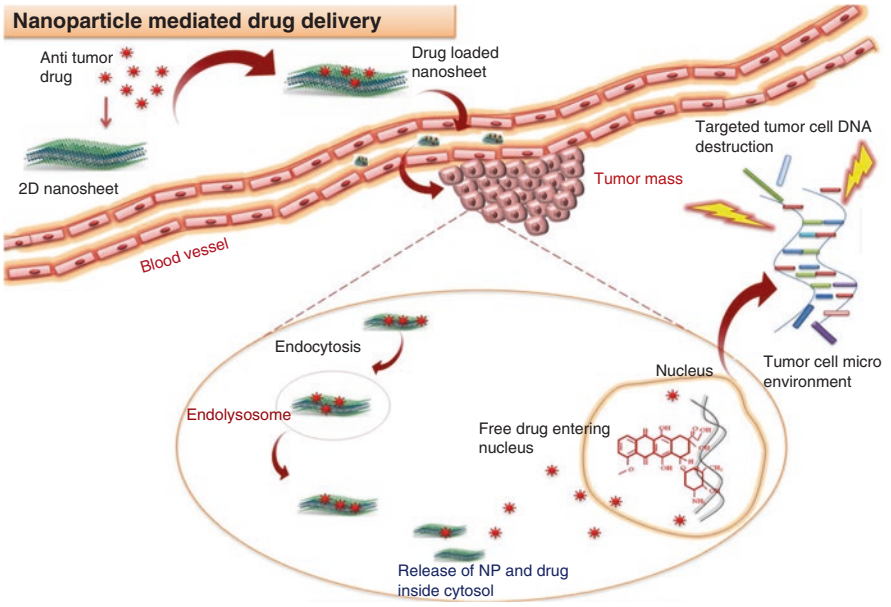

Fig. 19.2 Nanoparticle-mediated drug delivery

These are then targeted to the site of action via targeting moieties functionalized over the NP surface. Nowadays, combinatorial therapies involving imaging-guided drug delivery along with photothermal therapy and radiotherapy are under investigation for improved tumor theranostics. RFA is a novel therapeutic strategy used in the treatment of tumor which uses an imaging-guided needle electrode introduced on to the tumor site either through the skin or directly through surgery. This was followed by passing through a high-frequency electrical current, which creates hyperthermia to kill the tumor cells. This strategy can be effectively correlated with nanomedicine to propose a painless approach in cancer treatment. Currently gold NPs have been used in this field for noninvasive destruction of tumor cells. Optical properties exhibited by nanoshells can be used for tumor ablation technique using NIR light. One such kind was developed by Laroui et al. (2013) which uses nanoshells coated with gold NPs to absorb NIR radiation to specifically kill tumor cells.

Inorganic NPs like gold, silver, cerium, iron, TMDCs and their oxides and quantum dots and organic NPs like graphene, carbon nanotubes, fullerenes and chitosan have been widely researched for these purposes. Magnetic-activated cell sorting (MACS) with antibody-conjugated magnetic NPs can be used for specific antigen detection as a diagnostic tool. SERS (surface-enhanced Raman spectroscopy) technique using NPs can be used for sensitive immunoassay-based diagnosis. One such technique was developed for hepatitis detection using SERS active substrate incorporated into a microfluidic device for the detection of virus with very low detection limit (Kaminska et al. 2015). Drugs needed to get targeted to the brain for

neurological disorders are often associated with a challenge of crossing the blood-brain barrier (BBB). NP-based drug delivery approach from polymeric NPs to liposomes facilitates the transport of specific drugs crossing BBB. Modern medicine is right now focusing on this strategy to effectively treat various neurodegenerative disorders like Parkinson's disease. Nanomaterials used for clinical diagnosis and therapy are selected based on their morphological characteristics like size, shape, surface chemistry, stability, biocompatibility as well as toxicity. Current status of nanotechnology in tumor diagnosis and therapy will greatly improve methods like minute quantity tumor-cell detection, tumor imaging and tumor therapy with reduced toxicity compared to traditional treatment modalities. Currently more researches are focused on tumor therapy, but apart from that, dreadful conditions like neurodegenerative disorders as well as cardiovascular diseases are also recent targets of nanomedicine for improving precision diagnosis and therapy.

19.2.2 Imaging-Based Therapies

Contrast agents and imaging modalities are one among the basic necessities for advanced diagnostics and treatment in biomedicine. Current challenges in biomedical technology demand for the in-depth imaging of tissue microstructures and lesion characterization overcoming the existing tissue penetration limit and long-term toxic responses. NP-based contrast agents are under thorough research for various biomedical imaging modalities, including fluorescence imaging, MRI, CT, US, PET and SPECT. Compared to visible light, near-infrared fluorescence (NIRF) has advantages of deeper tissue penetration limit and less nonspecific tissue autofluorescence. Currently quantum dot-based fluorescence imaging researches are mainly focused for high-contrast imaging with deeper tissue penetration in order to enable high spatial resolution for disease diagnosis. QDs are having broad absorption spectra and narrow tunable emission spectra (Dutta et al. 2002). These kinds of NPs are designed so as to convert lower-energy photons to higher energy state to reduce blink and photobleaching effect. As a result, extensive effort has been made on the development of fluorescent nanoparticles for these purposes. In the last few decades, cadmium- and lead-containing QDs were largely investigated for these purposes, but now researches are more focused on synthesis of cadmium- and lead-free QD conjugates to avoid toxicity due to leaching effect.

TMDC-based and organic carbon-based QDs are therefore gaining acceptance in the recent times. Gold NPs have beneficial properties like good photostability, strong light scattering effect, excellent biocompatibility and ease of surface labeling which makes it a strong candidate for imaging-based diagnostics. Graphene is also having the same effects with high sensitivity. Likewise TMDC NPs are also having similar characteristics which can be well attributed for imaging-based theranostics. Nanoparticles for PET/SPECT are mainly used in tumor detection. Tumor imaging can occur through specific binding to receptors or via the EPR effect. Imaging can also be acquired through active and passive accumulation of NPs on the tumor site.

For this purpose, NPs can be specifically targeted to the site of action via coating with chemical as well as biological moieties like peptides, aptamers and antibodies. Copper, silver and gold NPs were greatly studied for PET-/SPECT-based diagnostic modalities. Ultrasound scanning is another application of NP-based imaging. Usually gold, silica and silver NPs are employed for these purposes which are specifically targeted to the site of action using various targeting moieties. In one such technique, when nanoparticles accumulate in the target tumor through active or passive mechanisms, the liquid already been occupied/filled in the nanoparticle core is stimulated by ultrasound waves to vaporize into gas to produce a strong acoustic reflection. This was proved with AuNP-coated/perfluorohexane-encapsulated/PEGylated mesoporous silica nanocapsules (Wang et al. 2013). With the advent of nanotechnology, single and precise imaging-based therapies have become more powerful, and multimodality imaging has got significant promise in the biomedical scenario.

19.2.3 Tissue Engineering and 3D Scaffolds

Tissue engineering is an interdisciplinary field integrating engineering, material science and medicine that aims to develop biological substitutes to repair, replace, retain or enhance tissue as well as organ-level functions (Hasan et al. 2018). Nanotechnology has got quite reasonable attention in tissue engineering. Very recently, nanoparticles have been used in tissue engineering in order to obtain improved mechanical and biological performances (Sensenig et al. 2012). The surface conjugation and conducting properties of gold nanoparticles, the antimicrobial properties of silver and other metallic nanoparticles and metal oxides, the fluorescence properties of quantum dots and the unique electromechanical properties of carbon nanotubes make them very useful in numerous tissue engineering and 3D scaffold applications. Scaffolds are three-dimensional biocompatible analogue of the extracellular matrix (ECM) of a given tissue, which provides a platform for cellular adherence, proliferation and differentiation. Scaffolds play a key role in tissue engineering through supporting the appropriate cellular activity without eliciting any undesirable local or systemic responses in the eventual host. Specially designed scaffolds enlighten the field of biomedicine as substitute for implantation of the bone, cartilage, ligament, skin, vascular tissues, neural tissues and skeletal muscle (Fig. 19.3). It was also found to be useful as vehicles for controlled delivery of drugs, proteins and DNA (Leijten and Khademhosseini 2016). Stem cells have shown appreciable blend with NP scaffolds for tissue engineering applications.

The ‘biomimetic’ scaffold mimics the properties of a native tissue which dynamically interact with the cell by generation and transmission of biophysical signals and undergo gradual replacement by newly synthesized tissue matrix. If it is possible to integrate the biologically complicated environment with engineering aspects of biomaterials especially NPs, it would be easy to make it practically applicable for biomedical technology (Yang et al. 2014). It has been reported that antibacterial nanoscaffolds were developed by introducing silver NPs to alginate/hyaluronic acid

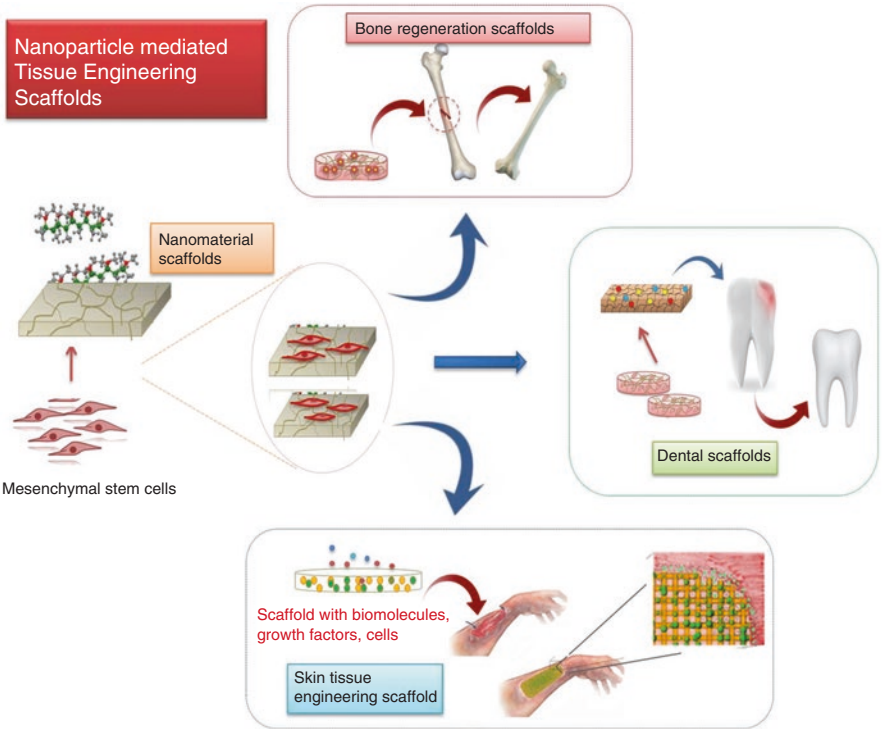

Fig. 19.3 Nanoparticles for tissue engineering scaffolds such as bone regeneration scaffolds, dental scaffolds and skin tissue engineering scaffolds

scaffolds which possess enhanced antimicrobial properties, beneficial in prosthesis implantation. Collagen/silver NP composite scaffolds were fabricated for skin tissue repair, especially useful for severe burn, whereas composite hyaluronan/silver NPs and hyaluronan/collagen-silver nanocomposite have found potent use as bone graft. Apart from silver nanoparticles, different metal oxide nanoparticles like magnesium, cerium oxides as well as their halogen compounds have also proven to be effective bactericidal agents. Metallic/polymeric NP composites are also gaining acceptance in recent times. Graphene and graphene oxide are another important candidates for tissue engineering applications and suitable for 3D bioprinting. 3D bioprinting ink can be made of graphene with a composition of graphene, solvent and an elastomeric polymer binder which makes it user-friendly and exhibit functional material properties (Syama and Mohanan 2019).

Various studies reported that MCF-7 cells and MSCs grown on graphene substrate will get attached and form a spindle shape with high proliferation and differentiation potential towards osteogenic lineages without the addition of any external biochemical cues, which can be employed for bone regeneration applications. Also electron spun PCL nanofibers coated with graphene oxide were developed for neuronal cell

regeneration towards oligodendrocytes with enhanced expression of myelin basic protein, Olig2, O4 and GalC (Shah et al. 2014). Likewise several researches are ongoing with NP-mediated tissue engineering and 3D scaffolds which are beneficial for organ regeneration and various other applications in the current medical scenario.

19.2.4 Biosensing

Nanotechnology is showing an increasing interest in the development of biosensors with high sensitivity and precision, especially in nanomedicine for the detection of specific bioanalytes (Singh et al. 2020). Because of their submicron size, nanoprobes, nanosensors and other nanoplatforms have allowed rapid and precise detection of target analytes in vivo. In order to increase sensitivity and to lower possible detection limits down to even individual biomolecules, nanomaterials are promising candidates due to its possibility to immobilize an enhanced quantity of bioreceptor units at reduced volumes and even to act itself as a transducer. Among such nanomaterials, gold NPs, semiconductor QDs, polymeric NPs, carbon nanotubes, nanodiamonds, fullerenes and graphene are intensively studied. As a result, portable instruments made of nanomaterials for the analysis of multiple bioanalytes are getting FDA approved and becoming commercially available nowadays. Within the group of noble metal nanoparticles, the most extensively studied one for biosensing application is gold NPs due to their high biocompatibility and excellent optical and electronic properties (Li et al. 2010).

Gold films have demonstrated their advantages in bioanalysis using surface plasmon resonance transduction. Another prominent example of nanomaterials used in biosensors is luminescent semiconductor nanocrystals called quantum dots, with the most studied category being cadmium chalcogenides. This makes use of fluorescence quenching technique for bioanalyte detection. FRET (fluorescence resonance energy transfer) with the aid of QDs has become an advanced methodology in biosensing applications. Another use of non-radiative energy transfer provoking QD fluorescence is the bioluminescence resonance energy transfer (BRET). Magnetic NPs are another alternative for fluorescent tags used in biosensors. It was reported that precise isolation of DNA strands from a complex media was achieved in a fast and efficient manner using silica- or gold-coated core/shell nanoparticles (Al Ogaidi et al. 2014). Graphene and carbon nanotubes are the most promising nanostructured carbon materials for biosensing applications where each allotrope has its particular advantage as transducer element (Liu et al. 2010).

19.2.5 Regenerative Medicine

Regenerative medicine is a new branch of medicine that deals with the repair, restoration and maintenance of the damaged tissues. Being a comparatively new field of biomedical research, most treatment strategies are still classified as experimental.

Studies have shown that regeneration of tissues can be made possible by a combination of living cells that provide the biological functionality and material scaffold, which will support the cellular proliferation. Diseases such as Parkinson's, Alzheimer's and osteoporosis are expected to be effectively treated in the nearby future by regenerating damaged tissues. Nanotechnology has got immense surge in the field of regenerative medicine which enables use of nanomaterials to improve the interaction between material surface and cellular environment that facilitates tissue regeneration. Cells are supported by extracellular matrix (ECM) that provides a supportive nutritive matrix for cellular microenvironment. This interaction between cell and ECM influence cell growth and proliferation and maintain the general homeostasis of the cell. Nanotechnology provides possibilities to produce scaffolds with nanoscale features that mimic the natural environment of the cell to promote cellular regeneration. The combination of nanomaterial fabricated with tissue engineering offers an ideal goal applicable for regenerative medicine (Fig. 19.4).

Ideally NP-conjugated stem cells are chosen as potent candidates for regenerative therapy owing to their ability to generate all types of tissue with their self-renewing capability. The functionalization of a stem cell-based nanomaterial porous scaffold with different biomolecules such as growth factors, gene and drug could enhance tissue engineering supported for regenerative therapies. Nanocoatings and nanomaterials can be used for this purpose to guide cell behavior along with the

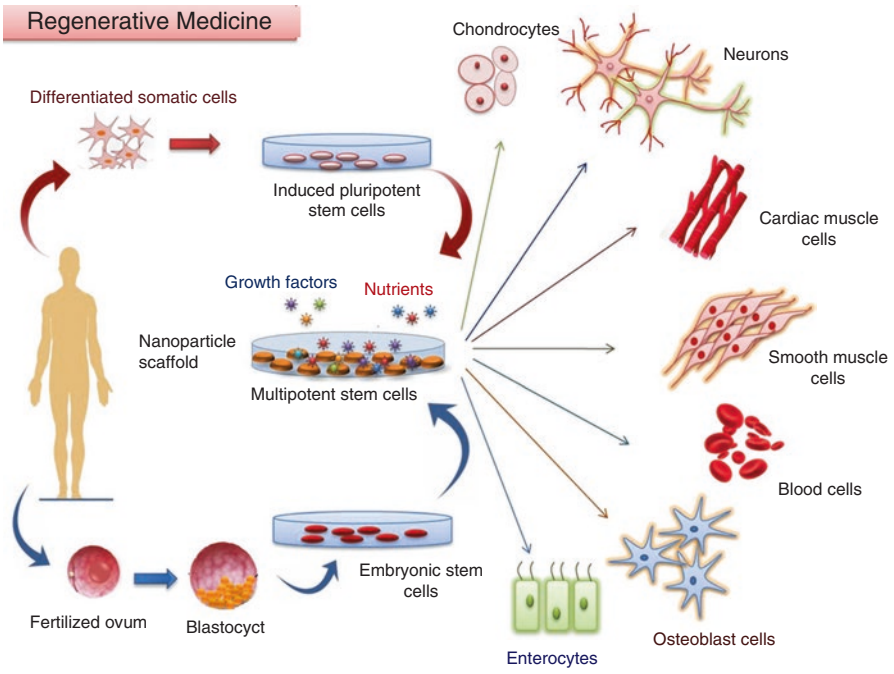

Fig. 19.4 Nanoparticle-mediated regenerative medicine

desired biological response. In bone regeneration, promising results have been obtained with ceramics and metallic nanomaterials facilitating increased osteoblast adhesion, cell proliferation and calcium deposition. Controlled delivery of biomolecules is crucial in tissue growth enhancement for regenerative technology, and several studies proved the efficiency of NPs for the same. NPs currently employed for these purposes include solid, hollow as well as porous metallic and polymeric microspheres, liposomes, micelles, microcapsules and dendrimers.

Commonly employed biodegradable polymeric NPs including polyethylene glycol (PEG), polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers in combination with hydrogels are proved to have excellent sustainable release profiles for controlled drug-gene delivery and regenerative tissue engineering applications. For urinary bladder, blood vessel and skin tissue regeneration, these degradable polymeric scaffolds were proven to be excellent candidates (Colombo et al. 2017). Titanium- as well as tentalum-coated nanophase apatites were investigated to promote bone growth and increased bone formation in vivo. It was reported that nanostructured PLGA, poly-ether-urethane and polycaprolactone are proven to be effective in bladder smooth muscle cell regeneration when compared to conventional methods (Thapa et al. 2003). Unique properties of graphene and carbon nanotubes have also currently been investigated as potent tissue engineering scaffold materials for stem cell-based regenerative therapy. In a recent study by Park et al. (2011), graphene scaffold was used as an inducer of neural stem cell (NSC) which differentiates into neurons. Another graphene-based nanomaterial was designed to guide rat neuronal stem cell differentiated into oligodendrocytes.

Cardiomyocytes and hepatic cell regeneration were studied in zebrafish using silver NPs, and suggestive results were obtained following in vivo administration as well. 2D clay NPs are the recent addition in the field of NP-mediated regenerative medicine which include the very recent member layered double hydroxides (LDH) and laponite. Studies reported that laponite gels display the ability to take up and bind bioactive molecules to direct the differentiation of endogenous cell behavior. Nanoclay materials like laponites were reported as materials which are able to sustain a localized regenerative microenvironment suitable for sustainable drug release as well as cell proliferation. Incorporation of anti-inflammatory and antimicrobial properties along with tissue engineering scaffold development could effectively enhance future application level strategies in the field of regenerative medicine to repair lost or damaged tissues.

19.2.6 Photothermal Therapy and Photodynamic Therapy

Photothermal therapy (PTT) is a minimally invasive technique which uses hyperthermia generated by photothermal agents to kill tumor cells. It mainly depends on triggering a photosensitizer using electromagnetic radiation such as microwaves, near infrared and even visible light to generate heat. This hyperthermic condition may trigger several processes such as protein/DNA degradation, membrane disruption as well as cytosolic exudation which lead to cell death. This phenomenon is

currently utilized by researches to kill tumor cells. Recently nanotechnology is being employed for these therapies. Inorganic and organic nano-photothermal agents such as gold NPs, carbon nanotubes, graphene NPs, quantum dots as well as TMDC NPs have currently been investigated for these purposes as they are excellent NIR photothermal agents. Among the reported 2D materials, TMDC nanosheets and black phosphorous have garnered most of the limelight in this field due to their easy tunable physico-chemical properties. Photodynamic therapy is a therapy which uses a drug or any chemical moiety called photosensitizer which when exposed to specific wavelength of light produces singlet oxygen species that kill the cells on which it is exposed to. Nowadays, researchers are more focused on NP-mediated photodynamic therapy to kill tumor cells. PTT and PDT together are used as a combinatorial therapy along with chemotherapy for improved cancer cell death (Fig. 19.5). Recently more and more researches are focusing on NP-mediated PTT and PDT efficacy which offers great potential in the modern medical scenario.

NPs for dual modal imaging-guided PTT/PDT combinatorial approaches have been recently investigated to improve treatment modalities for cancer (Wang et al. 2015). Synergistic PTT/PDT therapy is proved to be more effective than PTT followed by PDT. Naomi Halas and Jennifer West from Rice University were the key members who invented the first photothermal therapeutic NP in the mid-1990s.

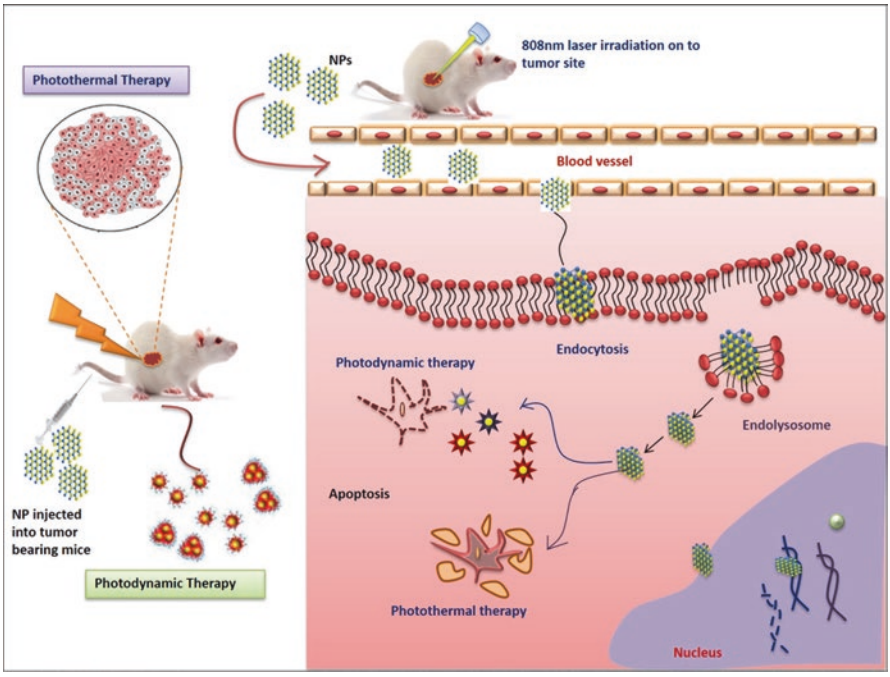

Fig. 19.5 Nanoparticle-mediated photothermal therapy (PTT) and photodynamic therapy (PDT)-targeted tumor destruction

They utilized PEGylated silica-cored gold nanoshell that has advanced into clinical trials as 'Auroshell' in 2008. Copper selenide nanocrystals are a recent member in the family of NP-mediated photothermal agents which exhibits a relatively strong NIR optical absorption. Hexagonal palladium nanosheets are another nanomaterial-based photothermal agent apart from conventional gold and silver NPs. Preclinical studies of NP-based PTT/PDT studies confirmed its successfully applicable potential in the near future by around the coming decade. Great challenges still exist while formulating these for application from benchtop to bedside. Thorough studies must be done before validating a safe nanoplatform for these PTT/PDT therapies because long-term in vivo toxicity effects are a suggestive possibility. Therefore long-term toxicity studies must be done to analyse the safest level of administration of NPs into physiological system. Also heavy metal containing and leachable NPs will be found it to be difficult for FDA approval.

19.2.7 Gene Therapy

Gene therapy has opened up unlimited potential to treat a wide variety of genetic disorders and has become a promising approach to replace chemical drugs and complicated surgeries in a more effective way. Vectors like virus and plasmid-mediated gene delivery have been researched from the past few decades, but current interest is focused on NP-mediated gene delivery. Lipid NPs like liposomes owing to their ability to fuse with biological membranes have been successfully employed to transfect DNA into cells and to deliver neurotransmitters and antibodies (Akbarzadeh et al. 2013). Various organic NPs such as carbon-based NPs like graphene, fullerene and carbon nanotubes and inorganic NPs like metals, metal oxides/sulfides and semiconductor NPs have found profound applications as gene delivery vectors due to their high surface to volume ratio, magnetic/optical properties and tunable surface modification possibilities. Chitosan was the first used self-aggregating complex for gene therapy in vitro. Metallic NPs like gold-, silver- and titanium-based gene delivery vectors have been studied to a greater extent in biomedical research. Among them, an extensively studied class is gold NPs like gold nanorods, nanosheets and quantum dots. Dendrimers are another class of organic NPs in which polyamidoamine (PAMAM) dendrimers are known to have the highest transfection efficacy among other such types due to the presence of enormous amine groups on their surface.

Generally primary amine groups participate in DNA binding, thereby promoting more DNA cellular uptake. These NPs are often times surface functionalized to prevent aggregation and provide with proper targeting effect to direct genes to specific sites for delivery. CRISPR/Cas9 gene editing system is one among the most reliable approaches for gene editing via DNA deletion, insertion and replacement with reports of more than hundreds suggesting its potent application in gene therapy. There are a growing number of manuscripts reporting several successful gene-edited cancer cell lines, but the real challenge is to translate this technique into

clinical practice. Apart from the conventional vectors, engineered NPs have been developed as relatively mature systems applicable to deliver CRISPR/Cas9 to the target cell. One recent study reported the efficacy of NP-targeted CRISPR-/Cas9-mediated genome editing in B cells in vivo. The selected NP was able to deliver CRISPR/Cas9 system to B cells and was able to induce Cas9 expression inside the cell and thereby disrupt B220 expression in B cells (Li et al. 2018).

19.2.8 Medical Robotics and Surgery

The envisioned nanorobotic applications span over a wide area of research from medical to environmental and defense applications. In biomedical sector, this emerging technology offers great potential, for example, nanorobotics could be used to give precise drug delivery for regenerative therapy as well as to cure inflammatory responses triggered by various agents. Scientific community has a stringent hope that this would cause a paradigm shift from treatment to prevention strategy in the current medical scenario. But most of the researches in this field still remain theoretical and underexplored to a great extent, primarily due to difficulties in fabricating such nanodevices. With nanotechnology, minute surgical instruments and nanorobots can be made which can be used to perform minute complicated surgeries at any part of the body. Instead of damaging a larger body part, these techniques could make use of precise and accurate targeting only in the area where the surgery needs to be done. Nanocameras facilitate visualization of surgery while the surgeon is performing the task which makes close-up visualization of each and every event during the surgery with accurate precision. With this technology, tissue, cellular as well as genetic level studies can be made possible. Future nanomedicine is expected to perform microsurgeries employing nanorobots injected into the patient.

Dentistry is one field in which nanorobots can have significant routine and specialized use in which these can be incorporated into almost every aspect of dental care including dentine hypersensitivity to orthodontics. A nanoknife with a 40-nm diameter has been developed and found to be effective for axon surgery (Saadeh and Vyas 2014). Another nanorobot consisting of engineered DNA strands that can autonomously detect cancerous cells and release treatment agents at the site has also been successfully developed. Once the site of action is reached, this DNA strand undergoes a structural reconfiguration that shifts from a closed to an open state releasing the stored therapeutics (Douglas et al. 2012). Also for screening and monitoring of life-threatening conditions, nanorobotics can be used especially intravascularly to analyse the progression of chronic diseases. Laproscopic cancer surgery is another appreciable possibility using nanorobots. Thus intravascular nanorobotics is a promising area of future healthcare scenario. Even though all these aspects seem to be fascinating, they could be realistic in the very nearby future itself.

19.3 Advantages and Disadvantages of Using NPs in Biomedicine

Nanotechnology has got a strong enough impact in the current medical research sector to improve the quality of medical research and healthcare with many reported success stories over the last several decades. Nanomedicine in a broad sense can be defined as the use of technology for comprehensive monitoring, repair and improvement of all biological systems from the cellular to molecular level with the aid of engineered nanodevices and nanostructures, ultimately nanorobots which are too small for the eyes to detect. Precisely saying, it would be of benefit in early diagnosis to effective treatment before prognosis of various life-threatening diseases without any side effects in a noninvasive manner. From disease diagnosis and theranostics to robotics, nanotechnology has revolutionized the biomedical technology towards a drastic extent. Nanotechnology often recommends with a future negative impact which is expected to appear in the next two to three decades of research and development horizon. Abrupt exposure of NPs with increased surface area often leads to a pressing uncertainty about how these particles behave in a perfect in vivo environment for a prolonged period of time. Increased chemical reactivity will tend to free radical production inside the cellular micro-environment which causes oxidative stress, inflammation as well as damage to biomolecules like DNA, proteins and nucleic acids which ultimately lead to severe toxic responses (Fig. 19.6).

Several unforeseen interactions could happen inside the cell that would lead to lot of unpredictable complications. According to the reported studies, NPs can accumulate in various tissues and organs over a period of time; moreover, it is unpredictable if it could effectively get excreted out. Furthermore, the ethical, social and legal facets of nanomedicine need to be tactically handled because there exist a lot of confusions regarding the possible risk factors behind once humans get exposed to it. Challenges arise once getting into clinical trials because ethical issues are a serious concern. Therefore it is imperative to make the society aware about the benefits and perils of nanomedicine.

19.4 Global Status of Nanotechnology in Biomedicine

Nanotechnology has progressed from a theoretical aspect to a rich area of proposals and ideas and now is an active area of practical research and developments. Nanotechnology has gained spectacular status across various biomedical and engineering disciplines due to their remarkable characteristics well opted for various reproducible strategies especially in biomedicine. It has got globally accepted as a next-generation technology for committed research in biomedical scenario to make remarkable achievements which can be made applicable from benchtop to bedside aspect. More than about 50 nanomaterial-based products are now FDA approved and are commercially available as drug formulations, sensors, imaging molecules,

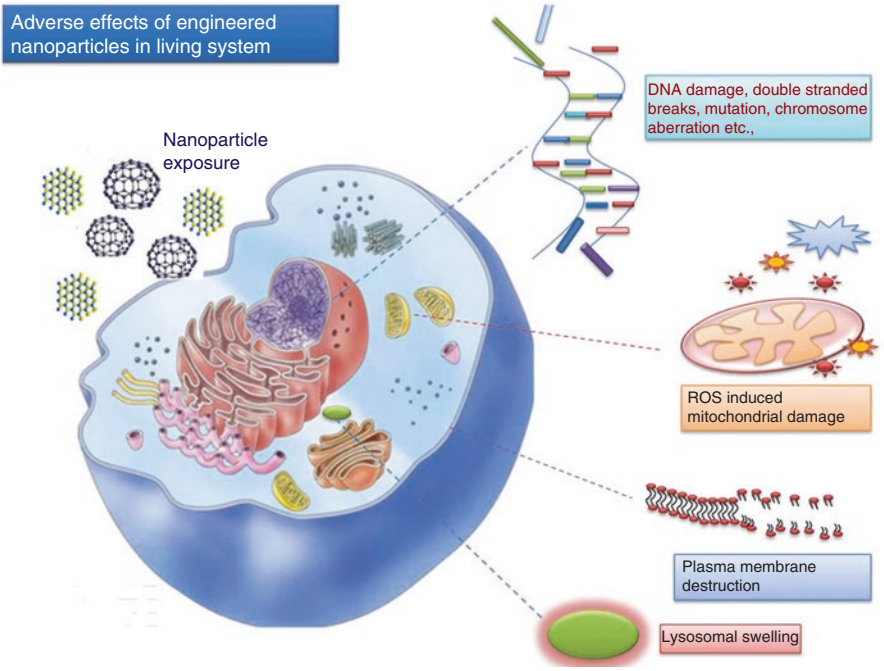

Fig. 19.6 Adverse effects of nanoparticles inside the living system

dyes, food nutraceuticals and other pharmaceutics. Globally a lot of researches are under progress with nanotechnology as the major aspect.

It was reported that researchers at Nagoya University are at the running time of developing a nanowire-based sensor to detect bladder and prostate cancer indicators in the urine sample, which hopefully will revolutionize medical sector globally. Also researchers at US San Diego are developing a method to analyse nanosized exosomes to check pancreatic cancer biomarkers. Osaka University Research Centre has combined nanospore sensors with artificial intelligence technique to identify single virus particles in biological sample. Another method for detecting cancer cells in the blood stream is being developed using NPs called nanoflares which will selectively bind to genetic targets in cancer cells and emit light when it binds with particular cancer genetic target. Thus researchers worldwide are developing NPs intended to make use in early detection of dreadful diseases like cancer and AIDS. One such method for early detection of brain tumor is under development with clinical trials which uses magnetic NPs and NMR (nuclear magnetic resonance) technology. In that, the researchers have used magnetic NPs attached to brain cancer cell-originated particles in the blood stream called microvesicles; the magnetic NP-conjugated microvesicle was then detected and quantified using NMR technology.

Nanotechnology innovation and commercialization have got a sudden hike in the last few decades. Between 1990 and 2008, about 17,600 companies worldwide

published about 52,600 scientific articles and applied for more than hundreds of patents in the nanotechnology domain (Moghimi et al. 2005).

Nanomedicine in Asian countries ranges from efforts to develop new possibilities using nanotechnology. High encouraging results have come from microfluidics-based nanosystem for in vitro imaging and processing. Korean Society for Nanomedicine used to develop nanomaterials and related technology virtually in a strategy of at least one every month. Nanotechnological researches began in India with the initiative of 'Nanoscience and Technology Initiative' with a 60 crore funding. In the year 2007, a 5-year program called Nanomission was launched with about 250 million USD funding, which spanned over multiple areas like basic research in nanotechnology, human resources development, infrastructure development and international collaboration. Now with the past few decades, India has contributed a lot in the field of nanotechnology and nanomedicine. India has published over 23,000 papers in nanoscience in the past 5 years and in 2013 was ranked third in the number of papers published in nanomedicine also, behind China and the USA. Now nanomedicine and technology have moved on to nanomedical robotics in surgery and nanosurgical instruments which offers tremendous output in the coming few decades. Researchers and medical practitioners have the hope that this could cause a paradigm shift from treatment to prevention of dreadful diseases in the medical community (Requicha 2003). But most of the researches conducted in this area remain still theoretical, primarily due to practical difficulties in fabricating such devices and lack of faith about its practical applicability.

19.5 Future Perspectives

Nanotechnology offers a myriad of potentials from sensing devices and energy conversion equipment to even life-saving bio-implants. Success on graphene and its derivatives has developed an equally impressive surge to search for other 2D materials which can effectively surpass the defects of graphene. The family of 2D materials comprises different chemical groups which include transition metal dichalcogenides, transition metal dioxides, 2D clay materials like layered double hydroxides and laponite, graphitic carbon nitride, hexagonal boron nitride, silicene and the very recent addition black phosphorus or phosphorene (Anju et al. 2019). These materials hold great potential in the biomedical field. Most of all being insoluble hinders its dispersity in physiological medium, but by proper surface modifications, stable dispersions of 2D materials can be made possible. Wide-size tunable band gap is one important aspect which makes them special over various graphene analogues. Hexagonal boron nitride also known as white graphene is a potent graphene analogue having a completely inert characteristic nature which is well known for its low fluorescence quenching property. The properties shown by 2D materials beyond graphene are sophisticated and distinct which makes them attractive candidates for novel biomedical applications.

Mainly their tunable band gap-dependant characteristics, unique chemical composition and low cytotoxic potential hold great promise for future biomedicine and therapeutics.

Gold NPs are currently under research for enhanced tumor killing effect due to its ability to enhance apoptotic effect on tumors; therefore radiation dosages can be minimized during combinatorial therapy of cancer. Quantum dots may be used in the future for locating tumors in patients and in the near term for performing diagnostic tests in samples. NPs can attach to proteins or other biomolecules, allowing detection of disease indicators in a lab sample at a very early stage. Likewise, there are several efforts to develop nanoparticle disease detection systems underway. Also for the early detection of blood-borne pathogens, peptide- or aptamer-conjugated NPs are gaining acceptance, and several researches on the same are undergoing in various parts of the world. Even though there will not exist a single unique nanomaterial to satisfy all the necessary requirements of biomedical scenario, the surprising possibilities of 2D nanomaterials provide an equally impressive surge to develop solutions for specific clinical challenges in the nearby future.

Acknowledgements The authors wish to express their sincere thanks to Director and Head of Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, for their encouragement and support for conducting this study. Anju thanks UGC, New Delhi, for financial support of Junior Research Fellowship.

Conflict of interest The authors declare that they have no conflict of interest.

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D. Thangadurai et al. (eds.), *Functional Bionanomaterials*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-41464-1