Sanjay Singh *Editor*

Emerging Trends in Nanomedicine

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About the Editor

Sanjay Singh is currently an Associate Professor at the Division of Biological and Life Sciences, Ahmedabad University, Gujarat, India. After completing his Ph.D. at CSIR-National Chemical Laboratory, Pune, India, he continued his research as a postdoctoral fellow at the University of Central Florida and the Pennsylvania State University, USA. His research interests include the synthesis of novel anti-cancer drug formulations and nanomaterials with biological, enzyme-like characteristics. He has received numerous national and international awards, such as an Endeavour Research Fellowship, YY Memorial Award, International Association of Advanced Materials Scientists Medal, Ambassador Australian Awards, and an EMBO Fellowship.

Dr Singh has published over 80 papers in international, peer-reviewed journals and authored and co-authored several books and book chapters. He has also worked as a guest editor for various annual meetings of the Society for Redox Biology and Medicine (SfRBM) and the World Molecular Imaging Society (WMIS). He is the Associate Editor-in-Chief of 3 Biotech (Springer Nature), OncoTargets, and Therapy.

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Introduction to Nanomedicines: Basic Concept and Applications

1

Ashok Kumar Jangid, Poonam Jain, Deep Pooja, and Hitesh Kulhari

Abstract

The application of nanotechnology in the medical field has grown significantly. Nanomaterials are being used for the diagnosis, prevention and treatment of diseases. This chapter is comprised of three sections. First section elaborates the concept and principle of nanomedicine. It also includes the advantages and disadvantages of nanomedicines. The second section discusses the applications of nanomaterials in bioimaging, drug delivery, nanozyme and biosensing. The last section is focused on the potential risks and challenges in the clinical translation of nanomedicines.

Keywords

Nanomedicine · Applications of nanotechnology · Potential risk and challenges

1.1 Basic Concept of Nanomedicine

Nanomedicine is an interdisciplinary science which involves the application of nanotechnology to health and medicine (Nezhadi et al. 2020). Nanomedicine involves various fields of science including chemistry, physics, engineering, medicine, and material science for diagnosis, control, curing, and treatment of diseases (Li et al. 2020a). In past two decades, nanotechnology has made a significant impact on the pharmaceutical formulation development. The unique physicochemical

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properties of nanostructures are responsible for the success of nanotechnology. Generally, a structure or a material containing at least one dimension in the size range of 1–100 nm is considered as a nanostructure or a nanomaterial. Because of this nanoscale size, nanostructures behave differently in comparison to its bulk form (Mirza and Siddiqui 2014). The physicochemical properties like particle size, size distribution, surface to volume ratio, surface energy etc. are significantly changed after conversion of a bulk material into a nanomaterial. Apart from physicochemical properties, a nanomaterial also has different optical, electrical and magnetic properties from its bulk material (Chang et al. 2015; Chi et al. 2020; Yamada et al. 2020).

When a therapeutic agent (drug or gene or other bioactive molecule) is encapsulated into a nanostructure, it is called nanomedicine. The development of a nanomedicine has several advantages over its conventional formulations (Guo et al. 2020; Shi 2020; Xue et al. 2020). The specific advantages of nanomedicines could be listed as follows: Nanomedicines

- Enhance the solubility, dissolution and bioavailability of the hydrophobic drugs.
- Prevent premature degradation of encapsulated molecules.
- Targeted nanomedicines enhance specificity of drugs towards diseased cells or tissue.
- Improve transport across the biological barriers and cellular penetration.
- Enhance therapeutic efficacy.
- Decrease toxicity of drugs by decreasing unwanted interaction with biological system.
- Provide opportunity to develop personalized medicines.

Various nanostructures like polymeric nanoparticles, lipid-based nanoparticles, metal or metal oxide-based nanostructures, dendrimers, and carbon-based nanostructures have deep explored for the development of nanomedicines (Afzal et al. 2019; Kopeček and Yang 2020; Wang et al. 2020b). Lipid-based formulation Doxil was the first nanomedicine, approved by FDA in 1996. It is a liposomal formulation of doxorubicin and used for the treatment of various cancers. After that, various nanoformulations have been developed and many are under clinical trials. Nanomedicines can be administered to the patient *via* almost every route of drug administration. The most commonly used routes for nanomedicines are oral, intravenous, intraperitoneal, transdermal, and nasal routes.

1.2 Applications of Nanotechnology in the Medical Field

The role of nanotechnology in various parts of medical field has been investigated. Different nanostructures have been used for bioimaging, drug and gene delivery, and as biosensors (Salata 2004). Metal or metal oxide-based nanostructures have also been explored for their nanozyme activities.

1.2.1 Bioimaging

Bioimaging means visualizing or obtaining the functional images of living biological systems and is widely used in biomedical field (Erathodiyil and Ying 2011; Sharma et al. 2006). It is mainly used in the determination of the health of tissues and monitoring of the progress of disease. Nanoparticles play an important role in the delivery of imaging probes either alone or in combination with therapeutic agents. Sometimes, nanoparticles itself act as guest nanoprobe and are used as bioimaging agent due to their unique imaging features (Tabaković et al. 2012). Owing to their specific magnetic, optical, luminescence, surface plasmonic, fluorescence, and radioactive properties, nanoparticles provide enhanced contrast in imaging process for early detection, diagnosis, and in image guided treatments (Li et al. 2014; Santra et al. 2004, 2005; Selvan et al. 2010; Sreejith et al. 2012).

Currently, several bioimaging techniques like X-ray, computed tomography (CT) scan, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), and fluorescence are being utilized to capture images of different anatomical areas of human body. These techniques are used for diagnosis of various diseases, pathogenic infections, and to determine the normal functionality of human body. Therefore, bioimaging is a visualization method in which the biological functions can be recognized non-invasively or can record this functional information from the living specimen (Yen et al. 2013b; Zhang et al. 2019).

1.2.1.1 Magnetic Resonance Imaging

Over the past years, MRI has emerged as the most promising biological imaging technique. It has high spatial resolution and ability to provide detailed anatomical information. MRI is a powerful, non-invasive diagnosis technique based on the soft-tissue contrast imaging in the real-time monitoring (Chen et al. 2012; Zhao et al. 2019). MRI technique uses large magnetic field and radiofrequencies to provide contrast images of various tissues of the body. Although, due to lack of sensitivity of MR imaging, contrast agents (CAs) are usually essential to improve the sensitivity of endogenous MRI by enhancing the MR signal (Jerban et al. 2020). In general, there are two types of MR CAs. (1) Positive CAs: these agents enhance the intensity of MR signal from the region in which they are present. Examples are gadolinium (Gd^{3+}) and manganese (Mn^{2+})-based CAs that produce a positive or bright MR image. (2) Negative CAs: these agents decrease the intensity of MR signal intensity and produce negative or dark MR images. Examples are superparamagnetic iron oxide-based CAs. These CAs can generate good anatomical images at very low concentrations (Avasthi et al. 2020; Deka et al. 2019; Xiao et al. 2016).

The inorganic nanoparticles have emerged as next generation MRI CAs. The use of nanoparticles based CAs as imaging probes has number of advantages over conventional CAs. Following features of nanoparticles, make them suitable CAs for MRI (Ni et al. 2017):

1. High chemical reactivity due to large surface area
2. Unique optical, electronic and magnetic properties at nanosize
3. Opportunity for surface modification with biomaterials
4. Good in-vivo stability
5. Significant cellular uptake and penetration

Gd, Mn and Fe nanoparticles have widely been investigated for their applications as CAs. Gd is a rare earth element and belongs to lanthanides. It has favourable contrast properties due to its paramagnetic behavior and has seven unpaired electrons in the 4f shell to provide good relaxivity (Craciun et al. 2017; Erstad et al. 2019). Gd or Gd-based nanoparticles have been studied for imaging of various cancer tissues (Gale and Caravan 2018; Gayathri et al. 2015).

For example, folic acid functionalized and β-cyclodextrin coated Gd oxide nanoparticles were developed to enhance MRI signals in the breast cancer induced BALB/c mice (Mortezazadeh et al. 2019). In another study, polymeric nanoparticles containing Gd-diethylenetriamine pentaacetic acid was used for diagnosis of liver cancer (Liu et al. 2011).

Although, Gd-based CAs show excellent MR signals but they also cause high nephrotoxicity. Therefore, in the past decade,lMn-based CAs have been highly encouraged for MR imaging due to their better biocompatibility (Briguori et al. 2006; Laissy et al. 2006; Ledneva et al. 2009). Recently, albumin stabilized MnO_2 nanoparticle were developed for MRI imaging of breast cancer. Mn_3O_4 nanoparticles showed ultrahigh relaxivity of water protons and provided T_1 weighed images with high contrast enhancement of the BALB/C nude mice with nasopharyngeal carcinoma xenografted tumors. Figure 1.1 demonstrates the potential of PEG coated MnO nanoparticles in MRI of renal carcinoma.

On the otherhand, SPIONs are used as negative contrast agent. Several scientific reports have shown the applicability of SPIONs as CA in MRI of various tissues (Thapa et al. 2017; Waddington et al. 2020; Yen et al. 2013a).

1.2.1.2 Fluorescence and Phosphorescence Imaging

Fluorescence/phosphorescence imaging is another promising technique that utilizes the emission properties of the imaging probes to visualize the object by optical microscopy (Baggaley et al. 2014; Sen et al. 2020). Several fluorescent agents like organic fluorophore, semiconductor quantum dots, luminescent lanthanides, carbon dots etc. have been developed for bioimaging purposes (Shcheslavskiy et al. 2016). Although, organic fluorophores have excellent imaging capability, they suffer with several drawbacks such as toxicity, non-specific binding with biological molecules and sometime unwanted quenching (Jahn et al. 2015). In comparison to organic fluorophores, nanoparticles are highly stable, biocompatible and less interactive with biological system. Further, nanoparticles can be targeted to a particular tissue or organ (Kritchenkov et al. 2020; Middha and Liu 2020; Xu et al. 2020; Zhen et al. 2021).

In the past few years, several fluorescent nanostructures have been developed for bioimaging purposes. Quantum dots are the most widely used imaging

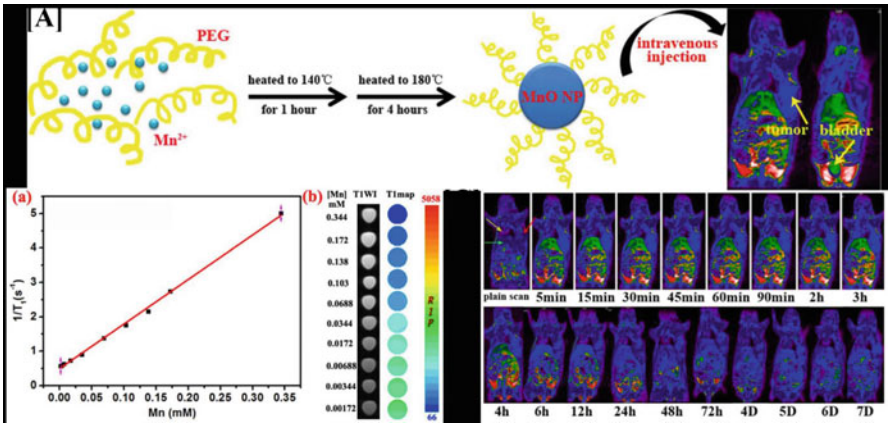

Fig. 1.1 (**A**) Scheme illustration of the one-pot preparation of hydrophilic PEG-MnO nanoparticles for magnetic resonance imaging of renal carcinoma in-vivo, (**B**) r1 relaxivity curve (**a**) and T1-weighted and T1-map MR images of PEG-MnO nanoparticles with various Mn concentrations (**b**), and (**C**) Pseudo-color MR T1 images of mice bearing renal carcinoma tumors pre- and post-injection of AS1411-PEG-MnO nanoprobe. The red, yellow and green arrows indicate tumor, heart and liver, respectively. Reprinted with the permission from Ref. Li et al. copyright © Elsevier

nanostructures (Chen and Yin 2014; Wolfbeis 2015). Quantum dots are bright, fluorescent nanostructures with specific properties of board excitation range, and size-dependent emission wavelength. Quantum dots reduce the photobleaching and remain stable for a longer time. Initially, quantum dots were synthesized using cadmium, selenium, and indium-based compounds. However, these QDs have been found to be cytotoxic to the cells. Therefore, these quantum dots were coated with biomolecules to reduce their toxic nature (Li and Zhu 2013; Singh et al. 2020; Venerando et al. 2020; Zhao and Zeng 2015). For example, glutathione capped CdSeS/ZnS core shell QDs were developed to obtain the images of MCF-7 human breast cancer cells. The novel core shell QDs showed time-dependent visualization of fluorescence within cancer cells, suggesting their applications in bio-labelling and imaging for cancer diagnostics (Fig. 1.2) (Rana et al. 2020).

Beside this, lanthanide-based bioimaging have gained much attention due to their high photostability, chemical stability, high luminescence quantum productivity and sharp emission bands. In addition, the optical properties of such nanomaterials can be further altered by doping and codoping. For example, in view of early cancer diagnosis, RE^{3+} doped CaF_2 upconversion nanocrystals (RE^{3+} = Ho, Tm, and Yb) were formed within the silica nanoparticles for detection of micro RNA, a common breast cancer biomarker. The results showed concentration dependent photoluminescence characteristic peaks (Wang et al. 2009; Ye et al. 2019).

Carbon quantum dots (CCDs) are excellent alternative of organic dyes and semiconductor quantum dots. These quantum dots possess several advantageous characteristics over organic dyes such as tunable emission, large two photon

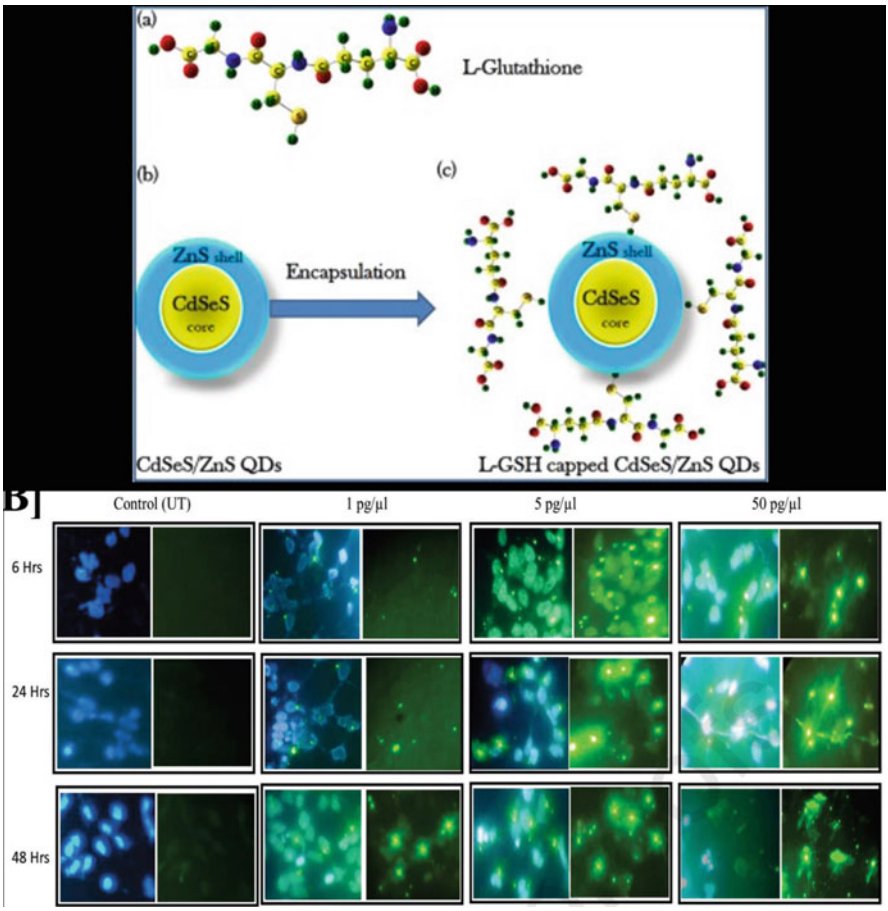

Fig. 1.2 (**A**) Scheme represent (**a**) Structure of L-glutathione (L-GSH), (**b**) Structure of CdSeS/ZnS QD probe, (**c**) Encapsulation of CdSeS/ZnS QD by L-GSH and formation of L-GSH-CdSeS/ZnS structure, and (**B**) Concentration and time dependent cellular uptake and localization of MCF-7 cancer cells with L-GSH-CdSeS/ZnS QD by fluorescent microscopy at 100X magnification. Reprinted with the permission from Ref. Rana et al. (2020) copyright © The Royal Society of Chemistry

excitation cross sections and high photostability. Owing to their biocompatible nature and excellent cell permeability, CCDs can be used as replacement for semiconductor quantum dots in various biomedical applications. For example, Yan et al. (2019), synthesized fluorescein functionalized CCDs for dual-emission ratiometric florescent ClO^{-1} biosensing and *in vivo* bioimaging (Fig. 1.3). The results of the study showed that the synthesized system had high sensitivity and selectivity for ClO^{-1} with low detection limit as 93 nM. The synthesized fluorescein

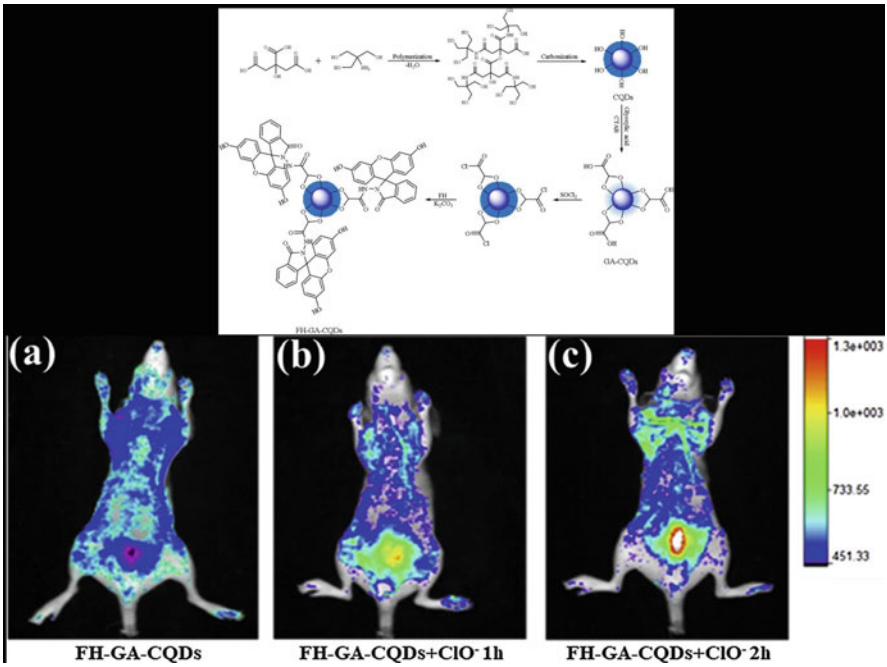

Fig. 1.3 (**a**) Synthesis Strategy of FH-GA-CQDs, and (**b**) Fluorescence imaging of the nude mice stained with 0.5 mg/mL of FH-GA-CQDs for 0.5 h in the absence and presence of 100 μM ClO^- for 1 h and 2 h. The excitation wavelength of 380 nm, collecting fluorescence intensity range: 460–630 nm. Reprinted with the permission from Ref. (Yan et al. 2019) copyright Elsevier

CCDs also showed low cytotoxicity, capability for sensing in both living cells and animals due to its dual emission property (Yan et al. 2019).

1.2.2 Drug Delivery Applications of Nanomedicine

1.2.2.1 Nanoparticulate Drug Delivery Carriers

Drug delivery means the designing of formulations and technologies for delivering of the therapeutic agents into the body. The carrier which holds the drug molecules is known as drug carrier. A drug carrier with a size of nanoscale range is known as nanocarrier. The delivery of drugs using a nanocarrier may offer several advantages over conventional drug delivery (Patra et al. 2018). These include:

- enhancement of solubility, dissolution and hence bioavailability of poorly water soluble drugs
- site-specific drug delivery
- can easily cross various biological barriers
- protection from drug degradation and improve stability

- possibility of surface modification for active targeting of drugs
- improve therapeutic index
- flexibility of route of administration

The commonly used drug nanocarriers are synthesized from polymers, lipids or proteins (Tagami et al. 2017). Polymeric nanocarriers include polymeric nanoparticles, nanomicelles, nanocapsules, nanospheres, core-shell structures and drug polymer conjugates (Khan et al. 2017; Qiu et al. 2020). The various forms of lipid-based nanocarriers are liposomes, niosomes, ethosomes, solid lipid nanoparticles, nanomicelles, lipid nanostructures, and drug-lipid conjugates (Neves et al. 2016). Protein nanoparticles, core-shell structures, and drug-protein conjugates are common forms of protein based nanocarriers. Metal/metal oxide nanoparticles and carbon-based nanostructures have also been investigated for drug delivery applications but only at research level. These nanostructures are generally discouraged at clinical level due to their severe toxicities and availability of better alternatives. Dendrimers are synthetic nanostructures and have been used for the delivery of various therapeutic agents (Kesharwani et al. 2015). Polymeric nanoparticles the most widely explored nanostructures for the delivery of drugs. Their stable structures, availability of different types of polymers, biocompatibility, and biodegradability make them suitable drug carrier (Zheng et al. 2020). The generally used polymers are either synthetic like poly(lactic-co-glycolic) acid (PLGA), poly(lactic) acid (PLA), poly(glutamic) acid (PGA) and poly(ethylene glycol) (PEG) or natural biopolymers such as chitosan, starch, inulin, dextran etc. (Fang et al. 2017; Houdaihed et al. 2018; Jangid et al. 2020). Different types of drug molecules like anticancer, antibacterial, antituberculosis, ocular drugs etc have been delivered using polymeric nanostructures (Kulhari et al. 2015; Pooja et al. 2015). However, high cost of the polymers and scale-up issue are their major limitations. Lipid-based nanostructures are the oldest and one of the most suitable drug carriers. Their non-toxic nature, high drug encapsulation capacity, feasibility of loading both hydrophilic and hydrophobic drugs, possibility of surface modification, and easy scale up and sterilization are major advantages of these nanocarriers (Farjadian et al. 2019). Like polymeric nanoparticles, a wide range of drug molecules have been successfully delivered using lipid-based nanocarriers. However, some of lipid-based nanocarriers (liposomes, niosomes, ethosomes) show physical instability after administration and release the encapsulated drug before reaching to its target site (Ahmad et al. 2018; El-Sawy et al. 2018). It becomes more concern for the drugs having narrow therapeutic index like anticancer drugs. Solid lipid nanoparticles which show high physical stability and sustained drug release, can help to overcome this issue (Nooli et al. 2017). Glyceryl monstearate, trimyristin, tripalmitin, oleic acid, stearic acid, stearyl amine etc are commonly used lipids for the preparation of lipidic nanoparticles. Protein-based nanostructures are generally prepared with both human and plant proteins. Various proteins like human serum albumin, bovine serum albumin, zein, β-lactoglobulin, casein, collagen, silk fibroin, gliadin and soy protein i.e. legumin have been used for the preparation of drug loaded nanoparticles (Ghosh et al. 2017; Tang et al. 2018). Being natural source derived, protein-based

nanoparticles biodegradable, metabolizable, biocompatible and can be easily modified with a targeting ligand on the surface (Oh et al. 2018; Zhu et al. 2019). Animal proteins are water soluble and need a cross-linking agent to convert it into a self-assemble spherical structure. Plant proteins are mostly water insoluble and nanostructure can be prepared by simple solvent evaporation or nanoprecipitation method. Further, plant proteins are cheaper than animal proteins. However, these proteins need to be sterilized before using to avoid the chance of infection (Tarhini et al. 2017; Defrates et al. 2018). Apart from polymer-, lipid-, and protein-based nanostructures, dendrimers mediated nanomedicines have also been designed and successfully translated into clinical nanomedicines. Dendrimers are synthetic 3D architecture with three basic units-central core, branching units and surface groups. Because of repeated branching units, various void spaces are generated with could hold the drug molecules. The commonly used dendrimers are synthesized from ethylene diamine, propylene amine, diaminobutane and amino acids. The nanoscale size, high monodispersity and surface functionality make dendrimers attractive carrier for delivering of different therapeutic agents. Further, multiple drugs can be encapsulated into dendrimersbased single formulation (Dias et al. 2020; Singh et al. 2017).

1.2.2.2 Targeted Nanomedicines

Drug targeting is an important and novel concept in the drug delivery. Targeting of a drug molecule to the diseased cells or tissue help to improve its therapeutic efficacy by increasing drug concentration in the diseased cells and reducing the exposure of the drug to the healthy cells. This controlled delivery can significantly reduce the total dose requirement and side effects. The importance of drug targeting is more for the low/narrow therapeutic indices drugs like anticancer drugs which show severe adverse effects due to non-specific biodistribution (Jeong et al. 2019; Mohan et al. 2018). With the evolution of nanocarriers, the possibility of drug targeting has increased tremendously. Drug molecules can be encapsulated into the matrices to nanoparticles and a targeting ligand can be attached on the surface of nanoparticles. The targeting ligand is an agent which directs the nanoparticles to the diseased site. For example, in case of cancer, there are several receptors which overexpress on cancer cells. So, a targeting ligand is the molecule which could bind to these receptors. Various molecules have been used as ligand for targeting the anticancer drug loaded nanoparticles. Some of those are small molecules like p-hydroxybutyric acid, peptides (bombesin, cRGDfK, angiopep), monoclonal antibodies (trastuzumab), vitamins (biotin, folic acid), proteins (lectins, transferrin), aptamers, and sugars (galactose) (Kadari et al. 2018; Kulhari et al. 2016; Tunki et al. 2019). The targeted nanomedicines specifically bind to the particular receptor being overexpressed on cancer cells, internalized through the receptor-mediated endocytosis process and release the encapsulated drug inside the cancer cells. Apart from cancer, nanoparticles have been designed for the delivery of drugs for the treatment of various diseases like cardiovascular, tuberculosis, brain diseases like Alzheimer, Parkinson etc., skin diseases, and ocular diseases (Debnath et al. 2019; Xiong et al. 2019). In addition to synthetic drugs, nanoparticles are being successfully used for

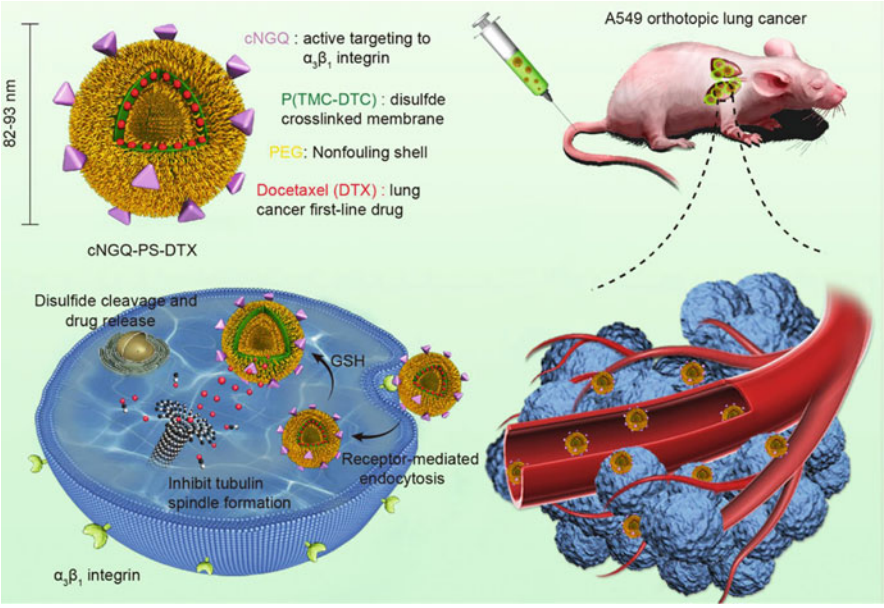

Fig. 1.4 Schematic represent the cNGQ-functionalized polymeric docetaxel (cNGQ-PS-DTX) nanoparticles for targeted delivery of DTX to α3β1 integrin over-expressing A549 human lung cancer. Reprinted with the permission from Ref. Zou et al. (2020) copyright © American Chemical Society

the delivery of natural therapeutic molecules like phytoconstituents-curcumin, resveratrol, morin hydrate, naringenin, EGCG, fisetin etc (Jangid et al. 2019; Radhakrishnan et al. 2019). Recently, Zou et al. (2020) designed docetaxel-loaded and cyclic cNGQGEQc peptide conjugated polymeric nanoparticles (cNGQ-PS-DTX) for lung cancer treatment (Fig. 1.4).

1.2.2.3 Delivery of Macromolecules and Genetic Materials Across Biological Barriers

Nanomedicines have also been designed for the delivery of therapeutic macromolecules like polynucleotides and polypeptides and genetic materials (SiRNA, DNA, mi-RNA) for the treatment of many diseases (Yang et al. 2020). However, the size and structural complexity of these macromolecules are the most challenging task to use them as a safe, stable and effective medicine. In addition, these macromolecules show fast elimination and poor metabolic stability after administration. These molecules have poor permeability across cellular membranes or epithelial tissues. The delivery of naked genetic materials show rapid degradation by the enzymes, poor permeability and rapid clearance. Therefore, the delivery of these macromolecules has been attempted using nanocarrier systems. The designed

nanoparticles not only stabilized and protected the macromolecules but also translocated across the barriers of epithelial tissue (Wen and Meng 2014).

Several two-dimensional and three-dimensional nanocarriers have been designed for their delivery of genes (Shehzad et al. 2016). Recently, Di Silvio et al. (2019) designed poly (allylamine hydrochloride) (PAH)-based nanoparticles for siRNA delivery. The designed nanoparticles were stable in physiological media and cell culture media but disintegrated at acidic pH of endosomes to deliver the encapsulated siRNA near to its target site i.e. nucleus. The results of the study showed that PAH/siRNAs were significantly effective in silencing GFP expression in A549 cells (Di Silvio et al. 2019).

1.2.2.4 Stimuli-Responsive Nanocarriers

Stimuli-responsive nanocarriers (SrNCs) are considered as the advanced and the most effective carriers to deliver therapeutic drugs at specific site without release of the drug during systematic circulation (Grzelczak et al. 2019; Li et al. 2020b). These SrNCs are degraded or release the encapsulated drug molecules in response to a particular stimulus. These stimuli may be endogenous (pH, enzyme, redox, temperature and light responsive), exogenous (magnetic field and ultrasound) or a combination of both (pH/enzyme, light/temperature and magnetic/redox etc.). The commonly explored SrNC are for the treatment of cancer. Cancer cells or tissues have different physiological conditions like slightly acidic pH, higher temperature, increased glutathione reductase level etc. from the normal cells or tissues (Chountoulesi et al. 2020; Li et al. 2019a; Mao et al. 2020).

Luo et al. (2020) developed cathepsin B/pH dual-sensitive block copolymer-based nanomicelles of doxorubicin for breast cancer treatment. The developed nanomicelles showed the pH and enzyme sensitive degradation. After the intravenously injection of the formulation in orthotropic and lung metastasis 4T1 breast cancer mice models, the stimuli-responsive-based antitumor and anti-metastatic effects were observed. The results of this study suggested the potency of the developed nanomedicine for breast cancer treatment (Luo et al. 2020).

1.2.3 Nanozymes

During the cellular metabolism in our body, several reactive oxygen species (ROS) including O_2^-, O_2^{-2}, OH, OOH, and H_2O_2 are produced. These ROS species are involved in many biological signalling pathways and interact with many biomolecules including DNA, RNA and lipids (Wang et al. 2019). The ROS generation plays important role in many biological processes like transduction, cell signalling, and body defence against pathogen invasion. However, the abnormal level of ROS can spoil the redox homeostasis which causes oxidative stress, damages of cell structure and change the function of macromolecules (Jiang et al. 2019). This over production of ROS causes several diseases specially neurogeneration, diabetes, atherosclerosis, aging etc. Therefore, enzymes like

superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase are protecting the cells from the oxidative stress and adverse effects of the ROS.

Nanozymes are the nanotechnology-based artificial enzyme which can be developed from various metallic, metal oxide and carbon nanoparticles. Nanozymes have promising advantages over the natural enzymes like scale-up production, low cost and high stability (Yu et al. 2020). Since 2004, many different nanomaterials have been explored for their application as nanozymes (Song et al. 2019; Wei and Wang 2013). The most commonly investigated nanozymes are synthesized from different metal or metal oxides composed of Fe, V, Co, Mo, Cu, Au, Mn, Ce etc. These nanomaterials have been investigated for their catalytic activity similar to the natural enzymes like superoxide dismutase (SOD), peroxidase, nuclease, catalase, oxidase etc (Fan et al. 2017; Li et al. 2019b; Wang et al. 2017). The Fe_3O_4 nanoparticles showed the oxidation of 3,3,5,5-tetramethylbenzidine (TMB), diazo-aminobenzene (DAB), and o-phenylenediamine (OPD) (Gao et al. 2007), suggesting that the Fe_3O_4-based nanozymes show excellent peroxidase-like properties because of presence of ferric and ferrous ions at the surface of nanoparticles. The surface of Fe_3O_4 nanoparticles can be modified by the different surface coating agent to enhance the performance of Fe_3O_4 nanozyme (Gao et al. 2007). Fan et al. (2017), synthesized histidine modified Fe_3O_4 (His@Fe_3O_4) nanozyme to mimic the structure of iron active sites with the horseradish peroxidase enzyme. The His@Fe_3O_4 nanozyme had significantly higher binding with H_2O_2 ($K_M >$ 10-folds) and catalytic efficiency ($k_{cat}/K_M >$ 20-folds) in comparison to bare Fe_3O_4 nanoparticles (Fan et al. 2017). Further, Weerathunge et al. (2019), developed quasi-cubic γ-Fe_2O_3 SPIONs with peroxidase like nanozyme activity and further conjugated with transferrin for the detection of brain cancer (Fig. 1.5) (Weerathunge et al. 2019).

1.2.4 Biosensing

Biosensing has emerged as a promising tool in the medical diagnostics to attain rapid and sensitive analysis (Holzinger et al. 2014; Ren et al. 2015). A biosensor device contains a biological receptor or unit with unique specificities to corresponding analytes such as DNAs, RNA, protein, enzymes, other body fluids, or potent diseased tissues of the living body. In addition, biosensor tools can also detect DNAs of bacteria or viruses or pathogens (Holzinger et al. 2014).

The integration of nanomaterials in biosensing has established a major advancement in the development of stable sensing probe and increase the signal capturing sensitivities. The unique structural properties of nanoscale-based materials such as high surface to volume ratio enables the immobilization of an increase amount of bioreceptors. In addition, the bio-functionalization of nanomaterials with biomolecules preserve the native property of nano structural materials and improves the site-specific application of biosensing devices. Nanomaterials which are used as biosensor are called nanobiosensors (Masteri-Farahani et al. 2021; Semenova et al. 2020). Nanobiosensors utilize the optical, chemical, magnetic or electrical property of nanomaterial to detect the biomolecules i.e. glucose, proteins, peptides, cytokines,

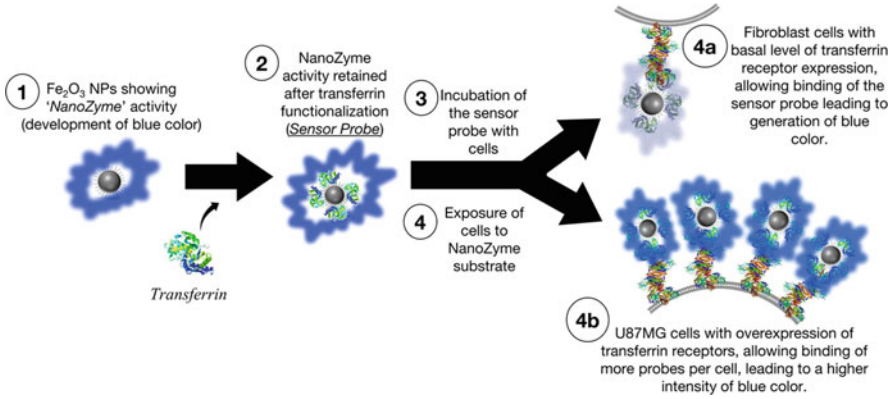

Fig. 1.5 Working principle of the colorimetric NanoZyme sensor illustrated through a Scheme outlining the steps involved during the sensing of glioblastoma (U87MG) cells. Step 1 shows the NanoZyme activity of SPIONs that are inherently capable of converting colourless TMB substrate to a blue coloured product in the presence of H2O2. Step 2 involves the covalent conjugation of transferrin (Tf) antibody to SPIONs such that the NanoZyme activity of SPIONs is not compromised. These Tf-functionalized SPIONs serve as the 'Sensor Probe'. Step 3 involves the exposure of the sensor probe to U87MG and fibroblast cells, resulting in a cell-SPION binding event where the magnitude of interactions depends on the cellular expression profile of TfR. Steps 4 involves the exposure of these cells independently to a peroxidase substrate. While fibroblast cells show basal level of TfR expression through a weak colorimetric signal (Step 4a), the overexpression of TfR on the surface of U87MG cells allows a significantly large number of sensor probes to bind to these cells leading to an intense tonality of blue colour (Step 4b). Reprinted with the permission from Ref. Weerathunge et al. (2019) copyright Elsevier

antigens, etc. The optical behavior of Au NPs is responsible for the surface plasmonic resonance and due to which a series of efficient colorimetric biosensors have been developed for the detection of oligonucleotide and DNA. For example, a novel Au NPs coupled with DNA super-sandwich were developed for the microRNA detection. Due to their surface plasmon resonance property and enhancement of refractive index of medium, the developed Au NPs showed a shift in the resonance angle, high detection sensitivity, selectivity to micro RNA and, resistant to matrix interference (Solaimuthu et al. 2020; Wang et al. 2020a). In a study, an ultra-high sensitive CDs/ZnO/PAN nanoparticles-based biosensor was designed for the detection of *Escherichia coli* (Pangajam et al. 2020).

CdSe/ZnS core shell quantum dots has been used as an electrochemical immunosensor for the detection of the extracellular domain of the human epidermal growth factor receptor 2, a human breast cancer biomarker (Cadkova et al. 2018). Quenching effect induced by nanoscale materials can be helpful in the development sensors for on-field applications. For example, graphene quantum dots were designed as label-free biosensing nanomaterial for the determination of dopamine and uric acid (Omar et al. 2016; Zhao et al. 2016).

1.3 Potential Risks of Nanomedicines

Nanomedicines have emerged as next generation medicines with several advancement in comparison to conventional formulations. However, there are also some concern regarding the use of the nanomedicines at clinical level. These potential concerns may limit their applications. The unique physicochemical properties of nanomedicines including size, shape, morphology, surface activity, stability, solubility and catalytic activity etc. influence their biological responses (Accomasso et al. 2018; Linkov et al. 2008). When a nanomedicine is injected into blood, the blood proteins or other blood components may easily adsorb on its surface and change its predetermined biodistribution pattern. Because of change in biodistribution pattern, nanomedicine may reach more to the non-targeted sites and cause adverse effects. An increase in size of nanoparticles may lead to uptake of nanoparticles by the reticuloendothelial system (RES) and high accumulation of nanoparticles in liver and spleen. Cationic nanoparticles with high positive surface charge are well reported to interact with red blood cells and cause hemolysis. Also, cationic nanoparticles show non-specific interaction and rapid uptake by the cells. The enhancement of endocytosis may cause potential risk of inflammation and pro-oxidant activity which are completely dependent on the surface charge of nanoparticles. Drug leakage before reaching to the disease or target site i.e. premature drug release has the potential risk of low therapeutic efficacy with high side effects. This is a common issue with some lipid-based formulations and nanomicelles. Micellar systems have another issue of stability. After administration into blood, nanomicelles may get dilute and lose their assembly. The concentration of the micelles forming agent may reach below to its critical micellar concentration and the assembly of micelles is disrupted. This loss of assembly results into complete premature release of drug (Bawa and Johnson 2007; Oberdörster 2010).

The nanomedicines significantly enhance the drug uptake and sustain drug release profile but the toxicity, stability and degradation of nanomaterials within the cells are still not reported well. Some nanocarriers have inherent toxicity to biological system (Resnik and Tinkle 2007). For example, carbon nanotubes are reported to cause pulmonary toxicity in mice and rats (Lam et al. 2004). Higher generation and amine-terminated PAMAM dendrimers are reported to accumulate into renal system (Albertazzi et al. 2013; Duncan and Izzo 2005).

Diagnostic nanostructures also have significant and potential risks (Borm et al. 2006). The repeated diagnosis leads accumulation of nanoparticles in body which causes serious side effects. Chen et al. (2015) investigated the toxicity effect of gadopentetate dimeglumine injection (GDI), SPIONs and manganese oxide (MnO) nanoparticles. From the observed results, the MnO and SPIONs were still safer in terms of toxicity while GDI showed high accumulation of Gd ions in liver, spleen and kidney which specifically induced the potential side effects (Chen et al. 2015).

High accumulation of metal or metal oxide nanoparticles may develop chronic diseases. The Gd-based MR contrast enhancer formulation showed induction of

nephropathy and nephrogenic systemic fibrosis. These agents are also reported to cause skin fibrosis and toxicity to other internal organs. Therefore, in 2017, FDA announced a new guideline for the use of all Gd-based MRI contrast agents and European Medicines Agency (Schieda et al. 2018) restricted to use five Gd-based contrast agents (Wang et al. 2018).

1.4 Challenges to Clinical Translation

In past two decades, the applications of nanotechnology in the medical field have been extensively investigated at both academic and industrial levels. A lot of success has been achieved in the use of nanoparticles in prevention, diagnosis and treatment of many diseases including several types of cancers, fungal infections, and ocular diseases (Azzawi et al. 2016). Several nanomedicines have been approved for their clinical uses and several formulations are under clinical trials. Even after successful translation of these nanomedicines, there are several challenges in translation of nanomedicines.

These challenges can be broadly classified at four levels: formulation level, pre-clinical level, clinical level, and commercial level. (1) At formulation level: development of a nanoformulation is different from the conventional formulation. A significant control on the physicochemical properties like size, dispersity, morphology, surface charge, stability etc. is needed. Being a new field, the availability of expertise in the development of nanoformulation is another challenge. Large scale production and reproducibility of drug encapsulation and other physicochemical parameters are further challenges at formulation development level. (2) At preclinical level: stability of the nanomedicine in the physiological fluids is one of the major challenges at preclinical testing. A detailed understating about the interaction of a nanomedicine with the biological system and its effects is required. Each nanomaterial may interact differently with the cells and tissues because of different surface chemistry (Hua et al. 2018). Sometimes, the nanoparticles prepared from the same material may behave differently due to change in size and morphology. (3) At clinical level: a very limited data are available about the behaviour and interaction of nanomedicines at clinical level. (4) At commercial level: High cost of production, small scale up, time-consuming processes, lack of clear regulatory guidelines and patient acceptance are major challenges in commercialization of nanomedicines (Satalkar et al. 2016). The major issues of nanomedicine formulation are shown in Fig. 1.6 (Metselaar and Lammers 2020).

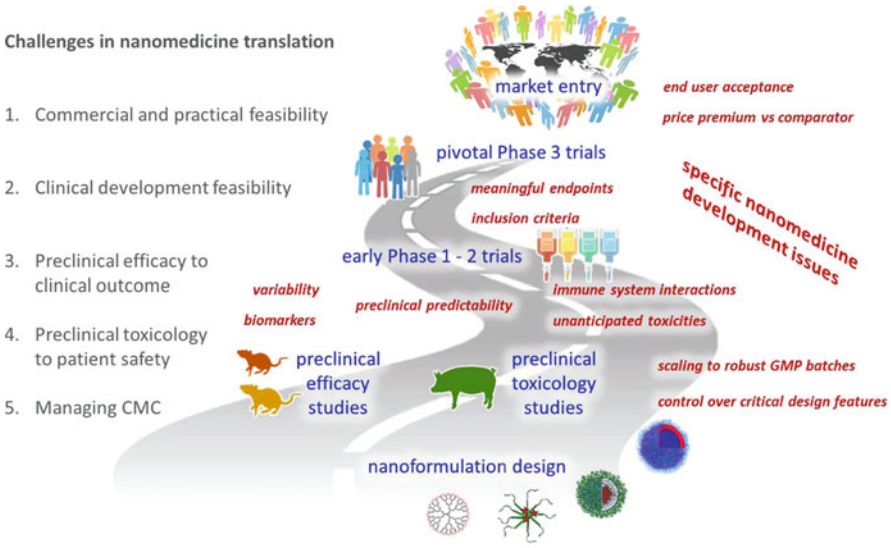

Fig. 1.6 Challenges in nanomedicine translation. Reprinted with the permission from Ref. Metselaar and Lammers (2020) copyright © Springer

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Strategies to Improve Oral Delivery of Natural Anticancer Molecules

2

Priyanka Shinde, Hina F. Badgujar, and Umesh Kumar

Abstract

Oral route of administration is the most accepted delivery route due to easy painless administration. Oral route is more suitable for chronic therapy and sustained delivery. In recent years oral approach has been used for delivery of various anticancer drugs like Zytiga, Capecitabine, Topotecan. The number of oral administrations of anti-tumour agents has been increased with respect to all other available cancer therapies. Preferable for patients as it reduces the efforts of regular visit to clinic or venepuncture. The most limiting factor for oral delivery is gastrointestinal barrier where epithelial membrane leads to poor permeability of drug and chemicals and enzymes in gastrointestinal tract causes degradation of bioactive compounds. In addition to this pH varies drastically across the GI tract, ranges from 1 to 2 pH in stomach reaching 7 to 8 in colon and rectum. To conquer this limitations nanocarriers are been developed to encapsulate and protect the drugs eventually increasing their pharmacokinetics. The most attractive method includes pH responsive soluble coatings, mucoadhesive nanocarriers, enzyme inhibitor and microbial control system. The present review discusses natural products in cancer treatment, obstacles in their oral delivery and nanotechnology approach to deal with the challenges.

Keywords

Oral delivery · Barriers · pH responsive · Mucoadhesive · Enzyme inhibitor

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2.1 Introduction

Drug delivery refers to wide range of techniques used for transport of therapeutic agents. Drugs are not administered in their native form but are converted into suitable formulation to improve its pharmacokinetics. The route of administration determines the suitability and effectiveness of drug delivery system. Among the various routes of delivery system in clinical arena, non-invasive route of delivery such as topical, intranasal and oral are chosen for delivery of drug. Among this, oral route of delivery is most widely used due to safety, conventionality and patient compliance. Also due to flexible dose schedule, reduced production cost and less men power demand, oral formulations are more preferable for pharmaceutical industries (Findlay et al. 2008).

However, physical, chemical and biological barriers in gastrointestinal tract (GI) remain critical challenge for effective oral drug delivery. First and foremost, the oral administered drugs are exposed to highly acidic pH and digestive enzymes in stomach and intestine (Goldberg and Gomez-Orellana 2003a, b). The mucus layer covering the epithelium contributes to poor permeability of drugs that needs to cross epithelial cell layer. Also, the tight junctions of epithelial cell restrict the paracellular transport. Moreover, for orally administered drugs, the dissolution depends on hydration rate and wettability of the tablet. This in turn depends on particle size distribution, shape and porosity of excipient material (Zhao and Augsburger 2005). Since the GI tract may lead to low bioavailability of orally administered drug hence it has become challenging to develop novel delivery system to overcome these limitations.

Recent advancement in the field of nanotechnology with respect to diagnosis and therapy has been useful for early detection of cancer, cellular and molecular imaging, and targeted drug delivery (Colomer et al. 2010). The nanocarrier system have been implemented for the improved shelf life, biodistribution and bioavailability of drug. However, a thorough understanding of chemical properties of drug and its behavior in physiological conditions is necessary for development of specific carrier system. As a result, much of attention is paid on the material properties of nanocarrier for enhanced activity of antitumor agents according to physiological requirements. Polymeric nanoparticles (NPs), lipid NPs, proteins, dendrimers, micelles, liposomes, carbon nanotubes and metallic particles are the presently used nanomaterials for cancer therapy (Byrne et al. 2008) (Fig. 2.1).

Several drugs like, Zytiga, Capecitabine, Topotecan are licensed for oral delivery. Paclitaxel oral nano formulation have shown 50% decrease in dose requirement compared to conventional available formulation. It has been demonstrated that orally administered Solid Lipid NPs are absorbed in intact form in GI and also internalized via clathrin-caveolae pathway (Li et al. 2009a, b). To enhance the solubility and bioavailability of compounds, Biorise technology is being used. This technology is based on the fact that since amorphous form of drug has higher solubility than its unmodified form hence drugs permeability and absorption rate get enhanced. Biocompatible carriers are mainly used in Biorise system that readily disperse in GI fluids (Marcato and Durán 2008). pH controlled system for oral delivery have also

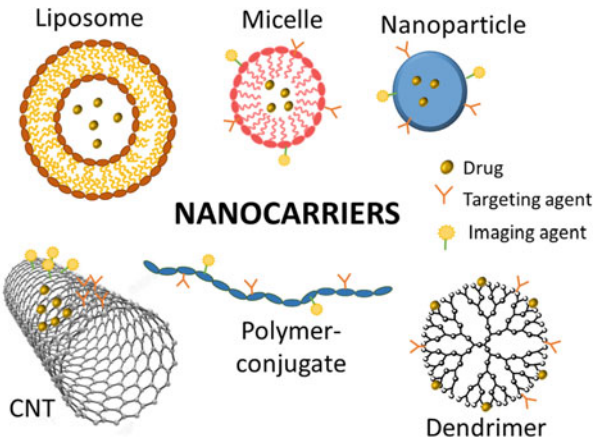

Fig. 2.1 Nanocarriers for cancer therapy

shown enhanced stability of drug in stomach and pH triggered release in intestine. Thus, a carrier system that disintegrates at specific pH has proved to be a reliable system for site specific delivery as specially for colon (Han et al. 2009). The functional groups on the surface of carbon nano tubes (CNTs) allow functionalization of wide variety of chemotherapeutic drugs on its surface. This CNTs have the property to cross membrane and transports the molecules without any toxic effect (Bianco et al. 2005).

This chapter discusses the challenges associated with oral drug delivery with focus on nano formulations approach to improve oral bioavailability of drug, natural product based currently available nanomedicine and future prospects.

2.2 Challenges Associated with Oral Delivery

With the rapid progress in biotechnology and bio pharmaceutics, large number of drugs have been approved by US Food and Drug Administration. Due to poor bioavailability of this drug product via oral and other alternative route, they are administered parenteral (Truong-Le et al. 2015). The oral bioavailability is affected mainly by aqueous solubility of drug, vulnerability in gastrointestinal tract, epithelium permeability, gut microbiota, drug dissolution rate and gastric transit time. The port for oral drug delivery is buccal cavity, oesophagus, stomach, small intestine and large intestine. The entire lumen is covered with a tough mucosal layer which acts as effective barrier for foreign molecules especially hydrophobic moieties. It is mainly composed of water and mucin protein; proteoglycan coating imparts it negative charge and lipid domain contribute to hydrophobicity (Lai et al. 2009; Leal et al. 2018). The thickness and composition vary according to the nutrient absorption rate and protective capability. Mucus may lead to structural change of proteins and peptides due to various interactions and transition of drug to gut associated lymphoid tissue is also affected due to inability of crossing the thick mucosal layer. Drug

efficacy may also decrease due to mechanical destruction and osmotic stress in GI tract (Ensign et al. 2012).

The pH is most vulnerable factor affecting stability of drug in GI lumen. It varies drastically from 7.4 to 6.2 of saliva to acidic pH 1–3 of stomach to neutral or slightly alkaline pH 6–7.5 in intestine. Administered molecules undergo oxidation, hydrolysis, deamination at these pH leading to lowering of their effectiveness or deactivation of drug (Moroz et al. 2016). Challenges for intestinal delivery include poor solubilization of drug at neutral pH. Moreover, in addition to pH; gastric, pancreatic and intestinal secreted enzyme may lead to degradation of biopharmaceuticals. The metabolizing enzyme include amylase for starch degradation, trypsin involving protein denaturation, lipase for fat decomposition and other. Also microsomal enzymes present in endoplasmic reticulum leads to metabolism of drug (Homayun et al. 2019).

The gastrointestinal tract of humans and other animals consists of diverse group of bacteria. Mostly ileum and colon has higher percentage of microbiota and comprises upto 10^{10}–10^{12} cells/g of intestinal content (Noh et al. 2017). The bacteria function symbiotically to ferment proteins and carbohydrate, produce vitamins, mediate immune response and thus are regarded as virtual metabolic organ that maintains human health and have impact on disease progression (Kang et al. 2013). Studies show that gut microbiota play important role in drug metabolism by chemical or enzymatic reaction that alter the pharmacological property of drug. The understanding of intestinal flora mediated modulation of drug efficacy can alter the systemic bioavailability of drug (Zhang et al. 2018).

The crucial step in determining drug bioavailability is its transportation through intestinal epithelial cells. This occurs mainly by paracellular transport (diffusion through space between epithelial cells), transcellular transport and membrane protein mediated transport. The transport of small hydrophilic molecule is permitted by paracellular route whereas large size hydrophobic molecules are transported transcellularly. Tight junctions in the epithelial cells are the major obstacles for absorption of molecules through paracellular route (Homayun et al. 2019).

2.3 Nanotechnology-Formulation Approach

Nevertheless, oral delivery is the most convenient route for delivery of pharmaceuticals in spite of above-mentioned challenges attempts have been made for the development of effective oral delivery system. Nanotechnology has been the future of the pharmaceutical industry and witnessed to enhance the aqueous solubility, physicochemical and physiological stability of various active pharmaceutical ingredients (APIs). The most convenient approach is to encapsulate into biodegradable nanoparticle that protect the drug from degradation and facilitate absorption of drug thus modifying the pharmacokinetic of active moiety (Fig. 2.2).

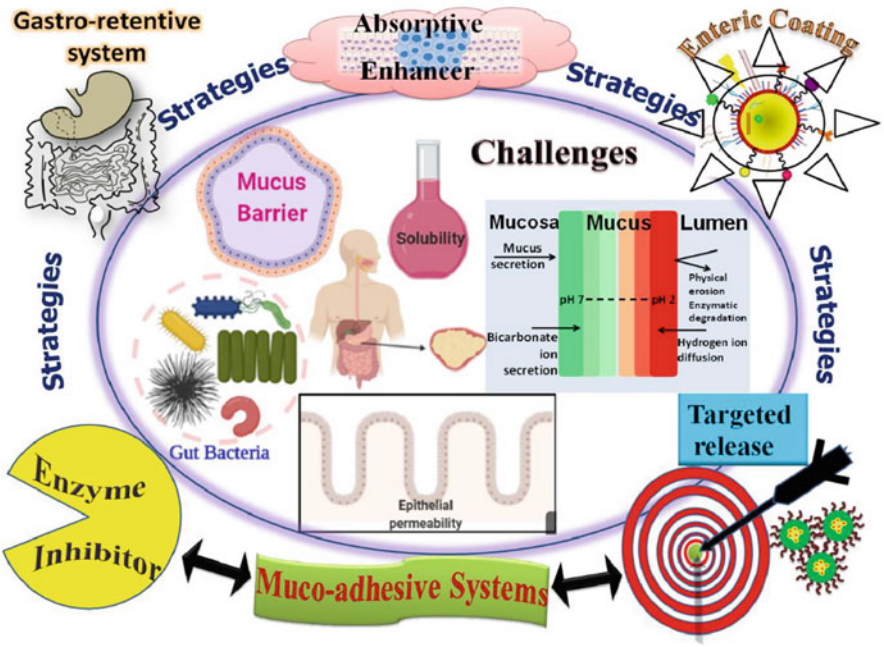

Fig. 2.2 Challenges of oral delivery and strategies to improve oral delivery system

2.3.1 Enzyme Inhibitors

Enzymes are biomolecules that act as catalyst in various metabolic reactions. Enzymes also act as catalyst in drug metabolism; mainly the hepatic and gut wall enzymes. Various drugs mainly peptides and proteins are easily degraded by gut wall enzymes before reaching to systemic circulations (Peters et al. 2016). The enzyme inhibitors are hence included in oral drug formulations to protect proteolytic inactivation of drug in GI tract and increase drug absorption. Leading enzyme inhibitors namely aminopeptidase inhibitors, sodium glycolate, soybean trypsin inhibitor, FK-488 and chymotrypsin inhibitors have shown to improve stability of orally administered drugs (Liu et al. 2003; Ueno et al. 2007; Verhoef et al. 1990). One of the major disadvantages of these inhibitors is that they may lead to absorption of proteins and peptides that are actually intended to degrade in the intestine. Also their use in long term may interfere with normal digestion and absorption of proteins and damage the structure and function of gastrointestinal tract (Bernkop-Schnurch 1998; Richard 2017).

Another approach to inhibit enzyme activity is utilizing the formulation components that modify local pH at site of enzyme activity (Baas and Thacker 1996). Most of the stomach enzyme are active at low pH whereas intestinal enzymes are active at neutral or slightly alkaline pH. Therefore, if the stomach pH is increased and intestinal pH is lowered then enzymes will be no longer active to degrade drug

molecule (Knarreborg et al. 2003). Certain polymers also inhibit enzyme activity and may be used to supplement activity of enzyme inhibitors. Polyacrylate and polymethacrylate inhibits elastase, trypsin and chymotrypsin. Similarly, carboxymethyl cellulose act as inhibitors of pepsin and elastase (Liu et al. 2013; Truong-Le et al. 2015; Yagi et al. 1980). Chitosan and its modified form are been widely exploited as enzyme inhibitors. Chitosan shows inhibitory effect towards trypsin, carboxypeptidase and aminopeptidase, however Chitosan-EDTA on Ca^{+2} dependent protease (Ways et al. 2018). Thiolated chitosan has been recently used for P-glycoprotein and cytochrome P450 inhibitors. Modified chitosan due to higher molecular weight have non penetrating characteristic that provides additional advantage of been not absorbed by GIT and remain concentrated on gastrointestinal membrane improving level of administered drug (Werle and Hoffer 2006).

2.3.2 Absorption Enhancer

The crucial stage in biopharmaceutical system development is absorption across the gastrointestinal tract membrane. The absorption of particles in GI tract highly depends on size and molecular weight of particle. Submicron size particles can be absorbed by transcytosis and for polymeric materials mainly paracellular transport takes place (Goldberg and Gomez-Orellana 2003a, b) (Fig. 2.3). The transport of polymeric nanoparticles owing to large molecular weight is difficult. A number of permeation enhancer have been used as functional excipient to facilitate enhanced absorption of orally administered drugs. Surfactants, bile salts, fatty acids, chelating agents, phospholipids, etc. can be included as permeation enhancer (Aungst 2012; Patel et al. 2011; Varma et al. 2003).

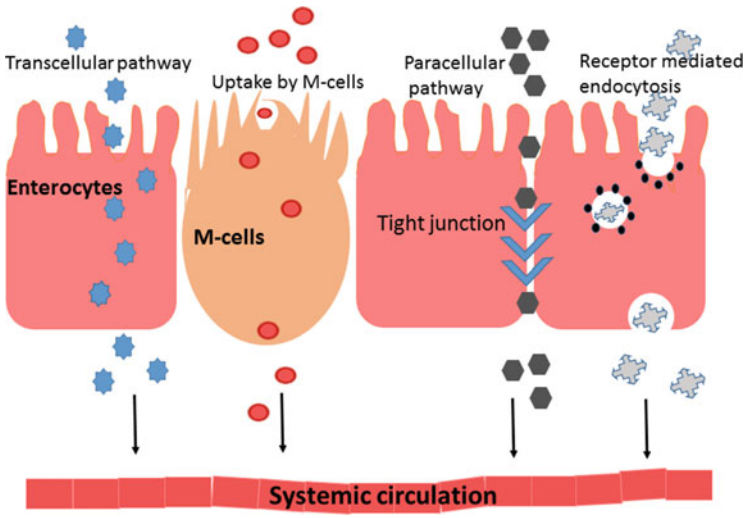

Fig. 2.3 Absorption pathway of oral drug

These enhancers act by modifying permeability of tight junction, enhancing membrane fluidity and by reduction in mucus viscosity. The Ca^{+2}chelating agents can disrupt tight junction proteins and have shown enhanced permeation of orally administered calcitonin (Mahato et al. 2003). Moreover, the wetting and solubilization property of surfactants has effective role in increasing drug absorption in GIT. Pluronic P85, Tween 20, Span 20, Brij 30 are few of the tested surfactants that effectively act as permeation enhancers. There was approx. 2.8 fold increase in rhodamine oral bioavailability upon administration with Myrj 52 (Föger et al. 2006). Also, few of the surfactants like Labrasol act via opening the tight junction of intestinal epithelial cells. The use of Labrasol in formulation for intestinal delivery of mannitol has led to dose dependent increase in mannitol permeability (Sha et al. 2005).

2.3.3 Mucoadhesive Systems

The role of mucus in the GI tract is to provide continuous protection against foreign particles and lubricate the epithelium surface (Carvalho et al. 2010; Ensign et al. 2012; MacAdam 1993). In order to provide effective protection from foreign entities and absorption of nutrients from food, the pH and thickness of mucus layer varies throughout the GI tract. The gastric mucosa sustains a gradient from pH 1–2 to pH 7–8 over a mucus thickness of only about 200 μm (MacAdam 1993). Therefore, the nanoparticles-based delivery system should be developed in such a way that it can improve drug pharmacokinetics and/or targeting without disrupting mucus mesh spacing.

Nanotechnology is shown to bridge the barrier of biological and physical sciences by applying nanostructure materials at various fields of science, especially in nanomedicine and nano-based mucoadhesive drug delivery systems, where such particles are of major interest (Orive et al. 2004). Over the years, researchers are working towards the development of nanomaterials that exhibits mucoadhesive properties for continued drug release system for various types of epithelia, including buccal (Portero et al. 2002), intestinal (Artursson et al. 1994) and nasal (Fernandez-Urrusuno et al. 1999) (Fig. 2.4). The use of mucoadhesive polymers for the development of pharmaceutical formulations is due to its high MW of the polymer chain, rapid adherence to form strong intermolecular hydrogen bond with the mucosal layer; penetration into the mucus network or tissue crevices and biodegradable character without producing any toxic by-products in mucosal layer (Roy et al. 2009).

With varying molecular architecture and different polymers like, sodium alginate, sodium carboxymethylcellulose, guar gum, hydroxyethylcellulose, karya gum, methylcellulose, thiolated poly(acrylic acid), poloxamer, celluloseacetophthalate, hydroxy ethyl cellulose, poly(amidoamine) dendrimers, poly(dimethyl siloxane) and poly(vinyl pyrrolidone) poly(ethylene glycol) (PEG), retene, tragacanth and poly(acrylic acid) were widely explored for the development of formulations having mucoadhesive properties (Chen and Cyr 1970; Park 1983; Smart et al. 1984;

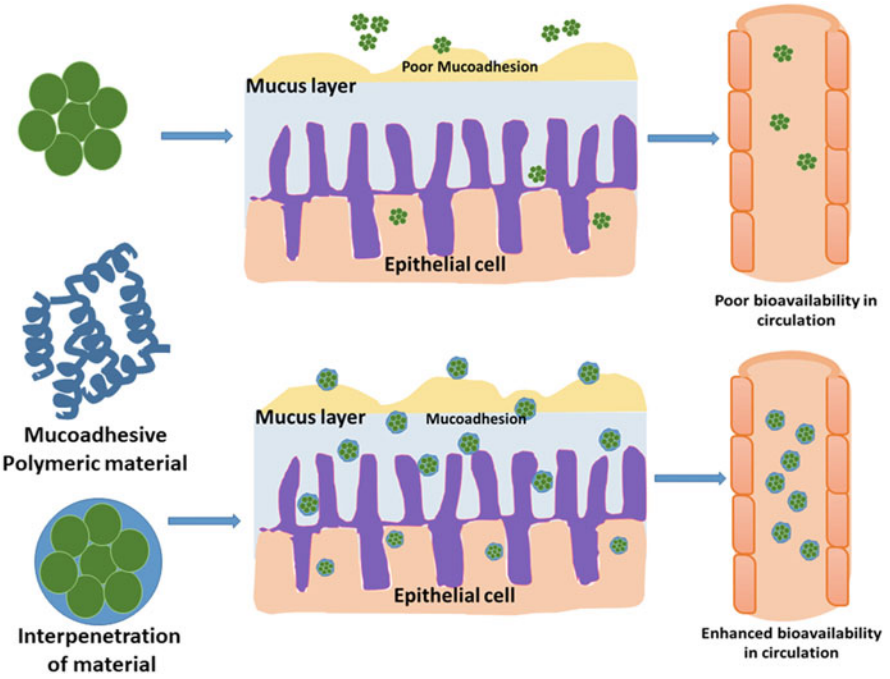

Fig. 2.4 Mucoadhesive drug delivery system

Sudhakar et al. 2006). Among this alginate biopolymer gel which has anionic property provides greater mucoadhesive strength compared to other cationic or neutral polymers.

Agarwal et al. have developed a pH dependent and microbially triggered drug targeting mechanism based on the conjugation of calcium alginate-carboxymethylcellulose (CMC) bead loaded 5-fluoroacyl (5-FU) and is targeted to the colon. The beads with lower CMC proportions presented greater swelling and muco-adhesiveness in the simulated colonic environment. With existence of colonic enzymes there was a 90% release of 5-FU encapsulated in the beads (Agarwal et al. 2015).

One research group has prepared a carrier composed of xanthan gum thiolated with l-cysteine to release tannic acid in the buccal mucosa to treat sialorrhea. Thiolation of xanthan gum resulted in increased adhesion on the buccal mucosa when compared to native xanthan gum. In addition, xanthan gum thiolate show increased salivary uptake capacity whereas tannic acid cause drying effect on buccal mucosa (Laffleur and Michalek 2017). Bhatia et al. has synthesized xanthan–mercaptopropionic acid conjugate (XMPA) and xanthan thioglycolic acid conjugate (XTGA) buccal pallet and study the in vitro release profile of metronidazole. The sustained release rate shows 8 h for XTGA while XMPA and XG buccal pellets could sustain the release till 5 h and 3 h only (Bhatia et al. 2015). Ni et al. embedded

cinaciguat nanocrystals in chitosan micro particles for pulmonary drug delivery of the hydrophobic drug. The nanoparticles were contrived for continuous release of the drug taking advantage of the swelling and muco-adhesive potential of the polymer. They found that inhalation efficacy might be conceded under the disease conditions, so more studies are needed to prove that this system has more potential (Ni et al. 2017).

2.3.4 Targeted Release

Another budding application of nanotechnology includes the development of tumor targeting especially in treatment of solid tumors. Nanoparticle scan target the cancer derived subpopulation by way of its commonly upregulated surface markers. The surface of nanoparticles can be decorated with specific ligands for targeting the receptor-mediated transport pathways. Furthermore, a successive nano based targeted drug delivery system (TDDS) allow particles for efficient uptake by a variety of cell types and selective drug rates (Kumari et al. 2010; Shi et al. 2018). Since cancer cells have faster growth rate than normal cells that require more nutrients and oxygen supply which can be obtained by the formation of new capillaries from existing blood vessels; termed as angiogenesis. There is an urgent need for a new comprehensive treatment strategy combining effective NPs with anti-angiogenesis therapeutic agents in the control of cancer. Studies have revealed that AuNPs show anti-angiogenic properties (Bhattacharya et al. 2004; Mukherjee et al. 2005). Versatile organic nanocarriers offer a number of advantages over their malleable spherical assembling properties, like solid lipid nanoparticles (SLNs), dendrimers, nano-emulsions, liposomes, polymeric micelles (PMs), virus-based nanoparticles (VNPs) and polymeric nanoparticles (PNPs) are highly stable, charged, form matrix to interact with drug compounds (Din et al. 2017).

Cancer cell lines established and maintained in vitro using tissue culture that are used to identify promising receptors for "active targeting" may not represent the properties of primary cancer cells found in a patient's tumor. The challenge, however, remains the precise characterization of molecular targets and ensuring that molecules only affect targeted organs. Over the past few years, progress in understanding of the mechanism of the nanoparticle uptake into the brain was made. This mechanism appears to be receptor-mediated endocytosis in brain capillary endothelial cells. Modification of the nanoparticles such as polymeric, dendrimer, silica and gold surfaces with covalently attached targeting ligands or by coating with certain surfactants enabling the adsorption of specific plasma proteins are necessary for this receptor-mediated uptake (Duncan and Izzo 2005; Wu et al. 2019).

Curcumin is an efficacious anti-cancer substance found in the cooking spice turmeric, but its clinical applications have been limited by its poor solubility and minimal systemic bioavailability. Bisht et al. have developed hydrogel nanoparticle system by encapsulating curcumin in a polymeric nanoparticle called "nanocurcumin". They have reported high dispersity of nanocurcumin in aqueous media which open up new opportunity to expand the clinical repertoire of this

efficacious agent by enabling soluble dispersion. It also showed similar mechanisms like free curcumin's on pancreatic cancer cells, including induction of cellular apoptosis, blockade of nuclear factor kappa B (NFkappaB) activation, and downregulation of steady state levels of multiple pro-inflammatory cytokines (IL-6, IL-8, and TNFalpha) (Bisht et al. 2007). Chemotherapy is extensively used to treat cancer, but it has drawback of multi drug resistance (MDR). Chen et al. have demonstrated the folic acid targeted nanogels for more efficient treatment of MDR cancer. They modified the surface of nanogels with polyethylene glycol (PEG) and FA-PEG via copper-free click chemistry to enable targeting of folate receptor expressing MDR cancer cells. The nanocarriers showed complete drug release within 24 h at mild acidic pH (pH 5). The nanogels can be internalized by receptor-mediated endocytosis and increase cellular uptake and cytotoxicity against folate receptor positive cells (Chen et al. 2017).

2.3.5 Gastro Retentive System

The most preferred and simple administration route of drug is oral delivery dosage among the various dosage forms developed so far for human administration. There are many parameters impacting the drug bioavailability of such pharmaceutical oral dosages. Among them the most important factors of physiological variability are the short gastric residence time (GRT) and unpredictable gastric emptying times (Kagan and Hoffman 2008). The approach to prolong gastric retention times and thereby improving the bioavailability of drugs will reduces drug wastage and also leads to site-specific drug release in the upper gastrointestinal tract (GIT) for local or systematic effect (Nayak et al. 2010; Rao et al. 2013). Various approaches have been made for the development of gastroretentive system which include mucoadhesive systems that causes bioadhesion to stomach mucosa e.g. super porous hydrogel system, magnetic systems etc. (Deshpande et al. 1997; Klausner et al. 2003a, b).

2.3.6 Enteric Coated System

Oral formulations intended for targeting various disease should possess several important features like, they should be easy to swallow, allow large drug doses which could be divided into small units, have the potential of combining various active ingredients in the same unit, offer good flow properties improving capsule filling and offer a fast gastric emptying time, decreasing the residence time in detrimental gastric fluids. Scientists have discovered enteric coating techniques to prevent drug release at gastric pH (Takenaka et al. 1980) and control release formulation that helps to attain the stability inside the GIT (Kojima and Nakagami 2002). Indeed, enteric coated pellets/sphere should accomplish smooth surface morphology, exhibit adequate hardness and friability, prolonged disintegration time, taste masker for bitter drugs. They attain single unit dosage (SUD) and multiple

unit dosage (MUD) to result in consistent drug release with reduced risk of local irritation and severity of adverse effects. The entrapment of drug substances into polymer-based microspheres or microcapsules has also become an attractive approach to mask the taste of bitter drugs by keeping the drugs from coming into contact with patients' taste buds (Chemtob et al. 1986; Fukushima et al. 2000; Singh et al. 2010). Okuda et al. have developed orally disintegrating tablets (ODTs) design by enteric-coated particles containing tamsulosin hydrochloride (TAM). They have physical mixed ODTs with rapidly disintegrating granules and micronized ethylcellulose at an appropriate compression force and prepared high tablet hardness, high physical stability, high resistivity against humidity, and rapid disintegration in the oral cavity (Okuda et al. 2014). Tummala et al. have formulated 5-fluorouracil (5-FU) enteric-coated nanoparticles (5-FUEC) using chitosan to target the drug in the colon area in a localized manner by preventing drug degradation in stomach and bypassing the systemic circulation resulting in enhanced anticancer activity in a sustained manner. They also evaluated for their enhanced apoptotic activity that may result in a consequently increased anticancer effect of 5-FU (Tummala et al. 2015).

It has been claimed that the coating should be highly elastic and flexible to be able to adapt to the deformation of the pellets during compaction. Lehmann and co-workers developed ODTs containing enteric-coated acetylsalicylic acid or indomethacin pellets coated with Eudragit® L and liberating less than 10%w/w of the active ingredient within 2 h in 0.1 M HCl. These tablets fulfilled the pharmacopeial requirements. They also concluded that inclusion of approximately 30% of excipients in the tablet formulation filled the interspace between the coated pellets, and thus separated the coatings, so that the tablets disintegrated rapidly with insignificant damage to the coatings and no notable change in the drug release. Premature drug release from enteric-coated dosage forms in the stomach, potentially resulting in degradation of drug or irritation of gastric mucosa, can be reduced with coated pellets because of more rapid transit time when compared to enteric-coated tablets. Soneja et al. have developed the pH-sensitive system for oral delivery of insulin. They checked the relative bioavailability of insulin in the enteric-coated capsule filled with chitosan/ poly(gamma-glutamic acid) and reported to be approximately 20%. From this in-vivo observation, it is clear that enteric coating protected insulin from the acidic environment of the stomach, thereby enhancing the intestinal absorption of insulin and providing a prolonged hypoglycaemic effect (Sonaje et al. 2010). Over the past few years, the enteric coated nanoparticles have significantly improved the stability, palatability, sustained-release performance and oral bioavailability. Liu et al. have developed a novel enteric pellet formulation with the goal of enhancing intestinal absorption efficiency of walnut peptides (WPs) (Liu et al. 2018). De Barros et al. have produced an enteric coated dried live probiotic pellet formulation suitable for delivery of viable probiotic *L. casei*. Release profile of *L. casei* was evaluated in vitro which effectively protected *L. casei* cells from gastric acid and achieved efficient distal intestinal delivery of viable cells (de Barros et al. 2015). These findings demonstrate the potential applicability of enteric formulations for oral delivery of acid-labile drugs. Solid lipid nanoparticles (SLNs) are a novel

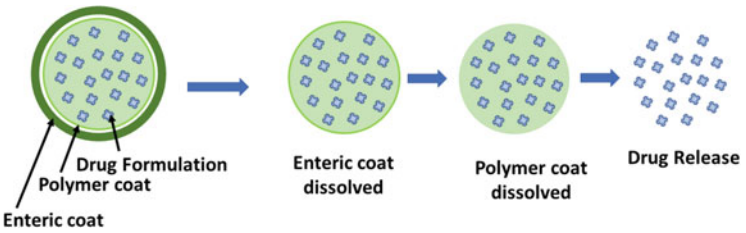

Fig. 2.5 Enteric coated system

nanosized drug delivery system using high-melting natural or synthetic solid lipids as backbone materials because of their good physiological compatibility and outstanding physicochemical property. Li and co-workers have formulated the enrofloxacin (ENR) enteric granules combining SLNs (lipid materials, emulsifiers, coating material) with enteric coating which significantly improved the stability, palatability, controlled release, and oral bioavailability of ENR. These results suggested that the combination of SLNs and enteric coating could be a way to increase the stability, palatability, sustained release, and bioavailability of other drugs (Li et al. 2019) (Fig. 2.5).

2.4 Natural Product-Based Nano Formulation

Phytochemicals are bioactive compounds found in natural products and have crucial therapeutic value due to less side effects compared to medicines. Lycopene from tomato, beta-carotene from carrot, glucan from oats, Omega-3 from fish oil are few of the extra nutritional bioactive compounds (Chen et al. 2006). This compound occurs in small quantities in foods and their presence in our daily diet play a crucial role in health maintenance. Fatty acids, vitamin C, polyphenols, flavonoids, carotenoids, etc. serve as antioxidants and provide protection against various diseases. Curcumin a well-known traditional medicine derived from plant rhizome has efficacy against respiratory disorders, inflammation, cancer and other disorders (Zaman et al. 2016). Animal studies have shown that curcumin prevents onset of carcinogenic tumor and also inhibit growth of implanted tumors. This has led to various clinical trials of curcumin in patients with multiple myeloma and different cancers (Dhillon et al. 2008; Sharma et al. 2004).

Nanotechnology has provided platform to overcome the challenges and barriers associated with solubility, stability, bioavailability and delivery of various compounds. Application of nanotechnology-based approach can provide an effective tool for delivery of nutraceuticals that follow the general principals of poor water-soluble drug. Studies have already been reported where nano formulation of herbal drugs and nutraceuticals are used for prevention of certain non-communicable diseases (Gunasekaran et al. 2014). The evidences of nano formulation and delivery system to prevent chemotherapeutic effect of natural products are still limited (Kashyap et al. 2019). Most of the nano formulation of phytochemicals are in

Table 2.1 Food active ingredients and type of cancer

Compound	Source	Cancer
Phenols	Fruits, parsley, celery, broccoli, cereals, tea	Squamous carcinoma, hepatic tumour, prostatic adenocarcinoma, pancreatic and myeloma cancer
Flavonoids	Citrus fruits, tomato, aromatic plants, berries	Brest cancer, hepatocellular carcinoma cells, glioblastoma, colorectal cancer
Carotenoids	Carrots, leafy vegetables, seafood, broccoli, pepper, apricot	Lung tumour, skin cancer, prostate cancer, colon cancer, gastric cancer
Essential oils	Flax seed, sesame seed, tea, medicinal plants	Prostate cancer, leiomyosarcoma, larynx carcinoma, gastric carcinoma, breast cancer
Vitamins	Carrots, nuts, fruits, seafood, dairy products, whole grains	Gastrointestinal cancers, prostate cancer, skin cancer
Minerals	Nuts, whole wheat, legumes, mushrooms, seafood, dairy products	Head and neck cancer, breast cancer, colon cancer

preliminary stages, the *in vivo* studies and clinical trials need to be performed for approval by Food and Drug Administration (FDA) (Bobo et al. 2016) (Table 2.1).

2.4.1 Nano Formulation of Phenolics and Polyphenolics

Phenolics are the secondary metabolites present in all vascular plants which were considered as waste metabolic products in past. Structurally they contain one or more benzene ring with hydroxyl group and may occur in simple to high molecular weight complex polymer (Munin and Edwards-Lévy 2011). A variety of phenolic compounds have been identified till date that lower the risk of health disorders due to antioxidant, anti-inflammatory, antiaging and anticarcinogenic properties (Bernal et al. 2011). Consumption of phenolics through fruits and plant food has several restrictions, also they are sensitive to food processing and storage that lead to unpleasant taste of phenols (Khoshnoudi-Nia et al. 2020). Their topical use is also limited sue to vulnerability to various environmental factors. They are prone to oxidation and hydroxylation and lead to appearance of brown colour (Fang and Bhandari 2010). Hence, there is a requirement of formulation that maintain the structural integrity of the compound until they reach to physiological target.

The utilization of phenolics in encapsulated form has been employed by many researchers for its stabilization. It has served to be the most promising technology for administration of phenols. Depending upon application different encapsulation methods are used that include emulsification, spray drying, fluid bed coating, liposome and electrospinning (Khoshnoudi-Nia et al. 2020; Mahdavi et al. 2014). Polymers of natural or synthetic origin and lipids are used as encapsulating materials. Among them, biodegradable polymers have attracted much interest due to easy designing, structural variation and good biocompatibility. Also, the biopolymer can be functionalized to achieve biomimetic character that serve to deliver

compounds directly at the desired site (Faridi Esfanjani and Jafari 2016). On the other hand, compounds such as casein and cyclodextrin have resemblance to extracellular matrix and can serve as good option of controlled bioactive system. Further apigenin loaded nanoparticles exhibit antitumor activity against squamous carcinoma and are also found to delay the progression of hepatic tumour (Bhattacharya et al. 2018; Rajendran et al. 2015). Another study suggested apigenin/resveratrol nano formulation have antiproliferative effect against SKOV-3 ovarian cancer cells (Nam et al. 2018). The *in-vitro* and *in-vivo* studies on liposomal encapsulation of resveratrol and curcumin showed apoptotic activation of p-Akt, cyclin D1 and androgen receptor in prostatic adenocarcinoma (Narayanan et al. 2009). Resveratrol encapsulation into dextran stearate micelles enhanced its cellular uptake in A549 cells, whereas resveratrol loaded lipid nano capsules mediated cell cycle arrest and apoptotic death in glioblastoma (Figueiro et al. 2013; Zhao et al. 2017). Resveratrol formulation containing Labrasol when administered orally showed dose dependent increase in its concentration in circulation (Zhou et al. 2015).

Activity and specificity of curcumin, the most potent ancient medicine has been increased tremendously in liposomal form. There is a significant increase in gastrointestinal absorption of liposomal curcumin with 70–80% inhibition of proliferative cell at lower dose (Kashyap et al. 2019). Studies have shown enhanced targeted delivery of curcumin in liposome to the prostate cancer cells (LNCaP and C4-2B). Curcumin loaded poly(lactic-co-glycolic acid (PLGA) nanospheres were successfully formulated and were able to affect cell viability of prostate cancer cells (Mukerjee and Vishwanatha 2009). PEGylated curcumin conjugate have revealed enhanced cytotoxic activity of curcumin towards pancreatic and myeloma cancer than free curcumin (Li et al. 2009a, b; Sou et al. 2009). Further, antiproliferative efficacy of curcumin encapsulated dendrosome showed substantial decrease in expression of SOX-2, OCT4 and Nanog genes (Tahmasebi Mirgani et al. 2014). Also certain natural product like capsaicin and piperine when co-administered with curcumin leads to higher availability of phenolic substance via oral route (Suresh and Srinivasan 2010).

2.4.2 Nano Formulation of Flavonoids

Flavonoids are low molecular weight compounds formed from phenylalanine and acetic acid derivatives. They are reported to inhibit cell signalling pathway, proliferation, invasion, apoptosis and angiogenesis in tumour tissues (Lin et al. 2017) and also attribute neurodegenerative, antiplatelet and antidiabetic activity (Survay et al. 2011). Among this antioxidant has been found to be the major activity of flavonoids that has attracted its application in food, nutritional and pharmaceutical industries. Anthocyanin has natural colorant property that can be used as dye in food industry but due to chemical instability and poor aqueous solubility its commercial use is limited. The intake of anthocyanin in fruits and vegetables has shown to prevent inflammation, heart disease and cancer. Nanoencapsulation of anthocyanin in biopolymer nanoparticle has attributed to its higher loading efficiency and efficacy

(Survay et al. 2011). Mucoadhesive gel of anthocyanin were formulated with raspberries to enhance penetration of anthocyanin into oral mucosa (Mallery et al. 2007).

Solid lipid nanoparticle of soy lecithin, glyceryl monostearate, tween and PEG has revealed best oral delivery system of poorly water-soluble flavonoids like quercetin. Liposome nano formulation of quercetin show anticancer effect against MCF-7 breast cancer cell lines. Quercetin phytosomes also have antiproliferative and apoptotic effect against MCF-7 cell (Minaei et al. 2016). In another study, quercetin phytosomes decreases mRNA expression of multidrug resistance associated protein (MRP1) (Sharma et al. 2018). The results of quercetin and ferulic acid incorporated protein and pullullan nanofibers showed sustained release of this compounds with improved anti-oxidant activity (Neo et al. 2013). Nanoliposome formulation of silibinin with glycyrrhizic acid has anticancer effect on HepG2 hepatocellular carcinoma cells (Ochi et al. 2016). Studies have demonstrated reduced cell viability and decrease expression of nuclear erythroid-related factor 2 (Nrf2) in breast cancer cells for phytosome containing luteolin (Majumdar et al. 2014). Coadministration of luteolin and silibinin inhibits glioblastoma cell growth through multiple mechanisms (Tsui et al. 2012).

2.4.3 Nano Formulation of Essential Oil

The cosmetic industry is paying much attention on development of natural eco-friendly products which are generally derived from the vegetable or seed oils (Balboa 2014). Oils are generally classified as triglycerides of fatty acids and may contain a small amount of hydrocarbons, alcohols, sterols, tocopherols or fat soluble vitamins. Essential oil (EO) are potential bioactive products having antibacterial, antioxidant, antiviral and anticancer properties. Theme, cinnamon, peppermint oil, carvacrol, lemon grass oil, thymol are few of the examples of EO having biological activity (Fernández-López and Viuda-Martos 2018). Most of the essential oils are volatile in nature and the vaporization can be minimized and shelf life can be increased by encapsulating process. Terpenes are the class of essential oils known for their anticancer effect. The bioavailability of celastrol was increased after loading into PEG block copolymer micelles in dose dependent manner (Li et al. 2012). Also, studies have reported antiproliferative activity of celastrol-polycaprolactone nanoparticles on prostate cancer cells (Sanna et al. 2015). Studies have shown that nature of coating agent has a major role in increasing the efficiency of certain oils. These components include gelatine, modified starch, maltodextrose and others. ω-3 PUFA found in fish oil is having anti-inflammatory property and protects against various cancers. It is insoluble in water and susceptible to oxidative deterioration causing rancidity and bad taste. Gelatin and sodium carboxymethylcellulose (NaCMC) can be used as encapsulating wall material to protect fatty acids and to improve use of fish oil in aqueous food systems (Bakry et al. 2016). Similarly, positively charged chitosan nanocarrier has improved mucus absorption of EO loaded nanoparticles and enhance the residence time and bioavailability of active

constituent (Bilia et al. 2014). Researchers have successfully encapsulated sunflower oil, palm oil, olive oil and other essential oils by means of various encapsulation methods to improve their antioxidant properties (Perona and Botham 2013; Roccia 2014).

2.4.4 Nano Formulation of Minerals

Calcium, magnesium, potassium, sodium, iron and phosphorus are the major minerals nutrients which are essential components of human health. They have role in various physiological and biochemical process including water and electrolyte balance, enzyme and hormonal function, have importance in bone, teeth and muscle strength and are integral part of biological membranes. This biominerals also possess aqueous solubility which is highly dependent on pH. At physiological pH they maintain crystalline structure that dissociates into ions or gases at lower acidic pH. Researchers have used this property to synthesized stimulus responsive carrier systems (Yang et al. 2005). Recently calcium phosphate (CaP) and calcium carbonate ($CaCO_3$) have been of much interest as biomineralized nanocarrier that selectively degrades at acidic pH of lysosomes, endosomes or tumors (Han et al. 2007). Mineral functionalized polymeric micelles and liposomes have shown the property of passive targeting and enhanced penetration that can be much effective in cancer treatment (Lee et al. 2010). Further magnetic nanomaterials have much progress in the field of biomedicine. They are mainly in superparamagnetic state and used for diagnostic purpose including MRI, tomography, NIR imaging (Stone et al. 2015). Superparamagnetic iron oxide nanoparticles (SPIONs) are mostly used as contrast agent in MRI and SPIONs liposomes are widely applied for delivery of chemotherapeutics agents for breast and colon cancer increasing their penetration (Augustin et al. 2016) (Table 2.2).

2.5 Future Prospects

The incorporation of bioactive compounds in food products provide a most effective way of developing functional foods for health wellness. However, the effect of these compounds depends on their bioavailability. Progress is made in designing the delivery system to boost the bioavailability in GI tract by use of absorption enhancer, enzyme inhibitors and other strategies. There is an existing gap in literature for toxicity risk of modified nano-system during long term intake. To predict the consequences of these systems in GI tract in more realistic manner in vitro digestive models needs to be designed, advanced analytical method to detect and quantify carrier should be developed and international standards for their toxicology should be confirmed. Also, in order to develop delivery system with increased efficacy and functionality, more attention needs to be given to understand the interaction between carrier matrix and bioactive molecule. Considerations should be given to multi drug resistance strategy of tumor cells and nanotechnology-based formulations containing

Table 2.2 List of nanostructured formulations of bioactive compounds

Bioactive components	Mechanism of action	Formulation/ delivery system	Efficacy/outcome
Curcumin	Inhibit cell growth by effecting various cellular pathways	Tween 80, soyabean oil and lecithin based microemulsion	Cytotoxicity against carcinoma and oral squamous cells
Curcumin	Apoptotic activity, induce cell cycle arrest at G0/G1 phase	Labrasol, Cremophor RH 40, Vitamin E TPGS and PEG 400	7–12 fold increase in oral bioavailability compared to pure drug
Rapamycin	Regulatory effect on cyclin D1 and c-myc cell cycle protein	Amphiphilic NMA622 nanoparticles	Reduction in tumor inhibition
Quercetin	Ability to scavenge high reactive species	SNEDD nano emulsion	Enhanced antioxidant activity
Quercetin	Inhibit enzymes responsible for activation of carcinogens	PEG-PE	Significant increase in solubility and antitumor efficacy
Tamoxifen	Possess antiproliferative action	PLGA nanoparticles	Increased bioavailability by 11 folds
Benzyl isothiocyanate	Detoxifies carcinogens. Disruption of microtubule polymerization in A549 lung cancer cells	Phosphatidyl choline and glycerol based nano emulsion	Increase permeability across Caco-2 cell lines and cytotoxicity against A549 cell lines
Emodin	Inhibit P13K and MAPK pathway	Poloxamer 188 and Tween 80 based nanoparticles	Increased cytotoxicity against MCF-7 and MDA-MB-231 cell line
Emodin	Effect on apoptosis related protein Bci-2 and Bax	TPGS liposomes	Cytotoxicity on K562 and L1210 leukemia cells
Ferulic acid	Antiproliferation and antimigration effect	PLGA microemulsion	Inhibition in human nin-small cell lung cancer cells
Apigenin	Modulate signalling pathway, triggers autophagy	Pluronic P123 and Solutol HS 15 nanoparticles	Enhanced antioxidant and anticarcinogenic activity
Luteolin	Inhibit cell migration and induces apoptosis	MPEG-PC nanospheres	Effective against breast cancer cells
Tea catechins	Modulate cancer signalling and metabolic pathways. Reduces reactive oxygen species	Chitosan and PLGA nanoparticles	Increased antioxidant activity and effective against Caco-2 cell lines
Triptolide	Inhibits cell proliferation and tumour metastasis	MPED-PLA micelles	Show antitumor activity
Artemisinin	Inhibits metastasis and angiogenesis	Phosphatidylcholine and cholesterol liposomes	Cytotoxicity against MCF-7 breast cancer cells

(continued)

Table 2.2 (continued)

Bioactive components	Mechanism of action	Formulation/ delivery system	Efficacy/outcome
Berberine	Inhibits P53 expression and induces caspase dependent apoptotic pathway	PEG liposomes	Cytotoxic against HepG2 cells
Noscapine	Alte steady state dynamic of microtubule assembly	HAS nanoparticles	Anticancer activity against HER-2 and SK-BR-3 cell lines
Thymoquinone	Induces DNS damage and inhibits telomerase	PLGA and PEG nanoparticles	Enhanced cytotoxicity against HCT-116 colon cancer cell, PC-3 prostate cancer cells

natural drug combination could be effective approach. There is also need of consideration of upcoming trend of personalized medicine that will significantly impact drug administration.

2.6 Conclusion

From ancient times bioactive compounds from plants have been used for prevention and treatment of various diseases. Majority of drug used for cancer treatment in past 2 decades have been derived from natural sources. Many of these compounds have low aqueous solubility and poor absorption profile in GI tract. Nanotechnology based oral delivery system modulated with different functional and physicochemical properties have shown to improve solubility, stability and permeability of natural bioactive molecules. These delivery systems are able to bypass various barriers of gastrointestinal tract and also targeting potential of such system is of major interest in cancer therapy. The oral delivery of natural anticancer agents via such nanocarriers will have a great impact on improving the quality of life of cancer patients. Also, the economic advantage of oral delivery system will significantly attract pharma agencies and can reduce the overall cost of health care.

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3 Nanoparticles Catalyzing Enzymatic Reactions: Recent Developments and Future Prospects

Nisha Yadav and Sanjay Singh

Abstract

Nanozymes are the nanoparticles which acts like natural enzymes and catalyze various biological reactions. Natural enzymes are facing a lot of issues in their applications such as expensive synthesis, lower stability, poor recyclability, sensitivity to pH and temperature, loss of activity on exposure to heavy metals etc. Nanozymes exhibit better catalytic activity than the corresponding natural enzymes even at the wide range of conditions of temperature and pH, hence they are better alternatives of natural enzymes. Nanozymes also offer high specificity to their substrate, easy synthesis, purification, and storage. Owing to these advantages, nanozymes have attracted tremendous attention of researchers to develop several applications of artificial enzymes in biomedical sciences. In this chapter, we have discussed different types of nanoparticles offering activities of biological enzymes. Based on the enzyme mimetic-activities, nanozymes are classified into three major groups, carbon-based nanozymes, metal-based nanozymes and metal oxide-based nanozymes. These nanozymes are further discussed based on the type of enzyme mimetic activities they display, such as Superoxide Dismutase, Catalase, Nuclease, Oxidase, Peroxidase, Phosphotriesterase, Phosphatase. These nanozymes are reported to be used as stable, highly efficient, robust and biocompatible catalyst, which can be used for the treatment of various enzyme-based disorders.

Keywords

Nanomaterials · Nanozymes · Enzyme mimics · Free radicals

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3.1 Introduction

Natural enzymes are proteinaceous in nature (except RNAse), which accelerate the rate of biochemical reactions in mammalian cells (Li and Breaker 1999). These biological enzymes are essential for the cellular metabolism and therefore for the life of an organism. Although the natural enzymes are crucial for life, they face a lot of intrinsic drawbacks such as globular shape, cost intensive synthesis process, complicated purification steps, and strict requirement of storage conditions etc. limiting their widespread applications (Lin et al. 2014). To overcome these issues, tremendous efforts are devoted to develop artificial enzymes, mimicking the activities of natural enzymes. Artificial enzymes are comparatively cheap, easy to synthesize and also offer good substrate specificity, therefore, used as an alternative to the natural enzymes (Breslow 2005).

In the early 1980s, a wide range of synthetic molecules have been reported as artificial enzymes such as cyclodextrins, palladium, and a composite of copper and curcumin etc. Breslow et al. have developed an artificial enzyme, based on cyclodextrins, which showed the function of ribonuclease enzyme by adding two imidazole rings to the cyclodextrins molecule (Breslow 1982). Naughton et al. reported that manganese (Mn) complexes with different ligands such as ethylenediaminetetraacetic acid (EDTA), and coupling agent, ethylenebis-(2-hydroxyphenylglycinate) (EHPG), exhibit superoxide dismutase (SOD) mimetic activity (Batinić-Haberle et al. 2010). SOD mimetic activity of Mn complexes was found to be dependent on ligands present in the medium, whether be it hydroxo, carboxylato or oxo/acetato. Palladium has also been used as a catalyst and functions as amino acid substitute (Loeb et al. 1998). Savadogo et al. developed palladium alloys, which can act as catalyst for oxygen reduction reaction (Savadogo et al. 2004). Copper-curcumin complex has also been reported as SOD mimic and can scavenge the superoxide anions (Barik et al. 2007).

Subsequently, several chemical and biological compounds are also investigated to display biological enzyme mimetic activities. Among them, nanoparticles (NPs) are also explored for exhibiting biological enzyme mimetic activities and termed as "nanozymes" (Wei and Wang 2013). The term "Nanozyme" was coined by Pasquato, Scrimin, and co-workers in year 2004 (Manea et al. 2004). They synthesized triazacyclonane-functionalized gold nanoparticles (AuNPs) and utilized them as a catalyst in a transphosphorylation reaction. This discovery inspired and attracted the attention of researchers from all over the world to develop novel nanozymes. The first nanozyme with peroxidase mimetic activity, was reported in 2007 by Gao et al. in ferromagnetic NPs (Gao et al. 2007). Since then, several attempts have been made to establish nanozymes as an effective alternative to natural enzymes and to improve their activity. These efforts include surface modification of nanozymes with different molecules, doping with efficient elements, and synthesis of hybrid NPs, etc. (Lou et al. 2019; Wang et al. 2019; Patel et al. 2018; Xi et al. 2019; Song et al. 2019). Utilizing these strategies, nanozymes not only display enhanced enzyme mimetic activity but also acquire multienzyme-like properties (Luca et al. 2014; Xia et al. 2015; Long et al. 2018; Jiao et al. 2018).

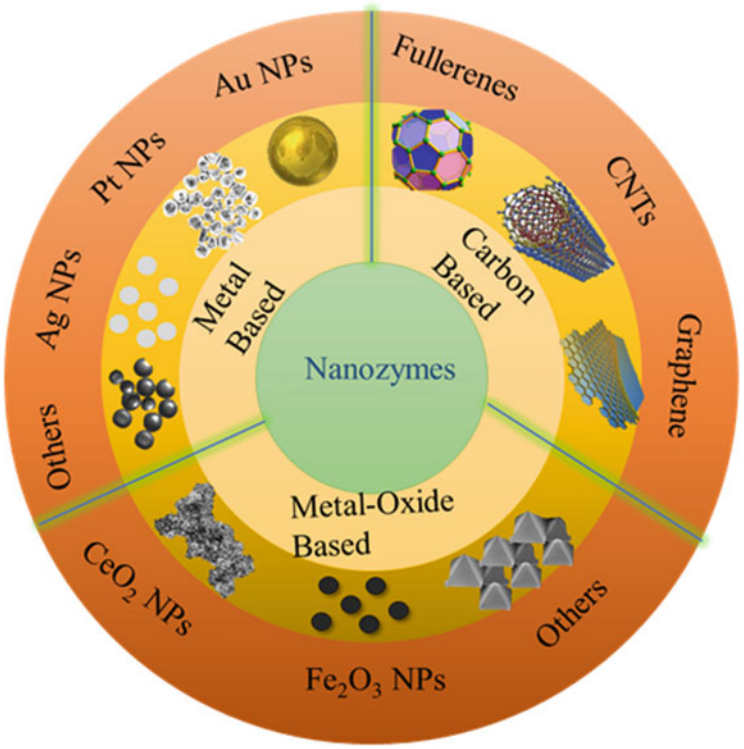

Fig. 3.1 Schematic representation of classification of various nanomaterials exhibiting catalytic activities of different natural enzymes

Nanozymes can be classified into three broader categories, carbon-based, metal-based and metal oxide-based nanomaterials (Fig. 3.1). These groups are discussed in detail in the further sections.

3.2 Carbon-Based Nanozymes

Carbon (C) is a versatile and unique element of the periodic table which has the potential to chemically combine with itself and almost all of the other elements (de Oliveira Penido et al. 2016) Due to its allotropy, carbon based materials exhibit several excellent properties such as high density, hardness, strength, and brittleness. These properties depend on the arrangement of adjacent carbon atoms. A variety of carbon NPs can be developed such as fullerenes, graphite, diamond, carbon nanotubes, graphene oxides and carbon dots (Maiti et al. 2019). There have been several biomedical applications realized from various forms of carbon, therefore, in the following section we will comprehensively present the catalytic properties of carbon-based nanozymes (Table 3.1).

Table 3.1 Table showing comparison of the different characteristics of natural enzymes and nanozymes

Sr. No.	Characteristics	Natural Enzyme	Nanozyme	Ref.
1.	Stability	Majority of natural enzymes are proteins, hence they face stability issues when they are exposed to extreme pH, high temperature, high pressure and exposure of proteases	Nanozymes, in contrast can respond to wide range of external stimuli and are able to perform their action in physiological environment as well as in harsh/changing environment	Longo and Combes (1998), Liu et al. (2019)
2.	Cost	Natural enzymes synthesis and purification steps are highly expensive	Nanozymes are much cheaper as compared to natural enzymes	Klein-Marcuschamer et al. (2012), Singh (2019)
3.	Synthesis	Manufacturing of natural enzymes is complex and time consuming process	Synthesis process of nanozymes is easy and of shorter duration	Alcalde et al. (2006), Motherwell et al. (2001)
4	Storage	Natural enzymes require specific conditions for storage and also cannot be stored at normal temperature for a longer period of time	Nanozymes can be stored at normal conditions (room temperature) and for a longer duration of time	Wei and Wang (2013), Wu et al. (2017)
5.	Recyclability	Natural enzymes are not recyclable	Nanozymes are recyclable	Wei and Wang (2013)
6.	Modifications	Modifications cannot be done with natural enzymes	Nanozymes has great surface area, so they can be easily modified by a number of ways like doping or capping	Liu and Liu (2017)
7.	Size effect	Enzymatic activity of natural enzymes is size dependent	Nanozymes exhibit size dependent activity, so we can obtain a range of enzyme mimetic activities by just changing the size or other properties of the NPs	Lin et al. (2014)
8.	Catalysis	Natural enzymes are good catalysts but under limited conditions (pH, temperature sensitive)	Catalysis function of nanozymes is comparable and in some cases even better than natural enzymes	Wennemers (2011)
9.	Reactivity	Natural enzymes reacts with their surroundings and lose their enzymatic activity	Enzymatic activity is less affected by surroundings as compared to natural enzymes	Liu et al. (2019)

3.2.1 Fullerenes and Its Derivatives

Buckminsterfullerene (C60) was firstly investigated by Harold Kroto and Richard Smalley in 1985. For this discovery, Kroto, Smalley and Curl were awarded for Nobel Prize in chemistry in 1996. Fullerenes, also known as bulky balls, consist of varying sized but even number of carbon atoms, which resembles like a cage or hollow sphere (Kroto et al. 1985). Fullerenes and their derivatives are among one of the early developed nanozymes because in 1990s, itself fullerene was found as nuclease mimic (Maiti et al. 2019). Subsequently, many beneficial features of fullerene were discovered including antioxidant behavior (ability to scavenge free radicals) (Chistyakov et al. 2013; Hu et al. 2012).

3.2.1.1 Fullerenes as Nuclease Mimic

Nucleases cleave the phosphodiester bonds of nucleotides in nucleic acids. They catalyze single and double strand break in the DNA (Deoxyribonucleic acid) which is one of the important machinery of DNA repair process. Defects in nucleases may lead to genetic instability and other types of immunodeficiency syndromes (Nishino and Morikawa 2002). Majority of the fullerenes and its derivatives are water insoluble which is the major issue for their application in biomedical sciences. Hence, to overcome this issue many methods have been adapted which involve the functionalization of fullerenes with hydrophilic moieties leading to improved water solubility (Herreros-López et al. 2017). Tokuyama et al. have developed water soluble fullerene (C60-1) and studied their ability to cleave DNA strands after photo-irradiation (Tokuyama et al. 1993). It was observed that C_{60}-1 could oxidatively cleave the DNA but with random cleavage pattern (C60-1) was not able to bind specifically to the site of DNA that need to be cleaved). Therefore, they synthesized a different fullerene compound (C60-2), which consists of 14-mer DNA sequence and was complementary to the target DNA (Boutorine et al. 1995). By this modification, specific cleavage at guanine rich sites was obtained. Other methods were also adapted to enhance the specific cleavage, for example fullerenes functionalized with acridine improved the DNA cleavage ability of the parent fullerene compound (Yamakoshi et al. 1996).

3.2.1.2 Fullerenes as SOD Mimic

ROS (reactive oxygen species) are highly unstable molecules which are produced in almost all aerobic cells. They are reduced by-products of mitochondrial metabolism and cellular response and they play important role in maintaining the body homeostasis as well as cell signaling. The condition of increased cellular levels of free radicals indices oxidative stress in cells that lead to damage in DNA, RNA (ribonucleic acid) and proteins. Superoxide anions (O_2^-) are one of the oxygen (O_2) free radicals generated as an end product of O_2 metabolism. Naturally, SOD enzyme is present in the living system that catalyzes the dismutation of O_2^- into hydrogen peroxide (H_2O_2) and O_2 (Devasagayam et al. 2004). The natural SOD enzyme face some limitations including low stability and expensive production mechanism which led to the development of artificial SOD mimics. Dugan et al.

have investigated the SOD mimetic activity of fullerenes (Dugan et al. 1996) and used them for neuroprotection after synthesizing polyhydroxylated fullerenes. Authors have also studied the radical scavenging ability of two different groups of polyhydroxylated fullerenes: Fullerene-2-ol (F-1 i.e. $C60(OH)_{12}$) and Fullerene-1-ol (F-2 i.e. $C60(OH)_nO_m$), where the value of n can be 18–20 and m can be 3–7 hemiketal groups. It was found that both of the fullerene derivatives could scavenge the free radicals and cause apoptosis in cultured cortical neurons (Dugan et al. 1996; Krusic et al. 1991). Further it was also investigated that fullerenes with C_3 symmetry (C60-C_3) were more effective in radical scavenging than corresponding control derivatives (Dugan et al. 1997). The SOD mimetic activity of methionine modified C_{60} was also studied on SH-SY5Y neuroblastoma cells. Results revealed that fullerenes exhibit neuroprotective effect and protection from oxidative stress, induced by lead ions (Chen et al. 2011). To demonstrate the SOD mimetic activity of C60-C_3 in living system, Dugan et al. investigated the therapeutic efficacy in SOD2 knockout mice (SOD2 is a manganese-based SOD enzyme found in mitochondria). They observed that the knockout model were able to survive for few days after birth but when C60-C_3 was administered the life span was increased by 300% (Ali et al. 2004) (Fig. 3.2).

3.2.1.3 Fullerenes as Peroxidase Mimic

Peroxidases belong to the oxidoreductase class of enzymes that catalyze the degradation of H_2O_2 in to H_2O and O_2. Okuda et al. have demonstrated the peroxidase mimetic activity of water soluble functionalized fullerenes, (C60-dimalonic acid, $C62(COOH)_4$). Water insolubility of fullerenes is one of the major limitations in terms of the biomedical applications, therefore, C60-dimalonic acid ($C62(COOH)_4$ was developed, which showed sufficient water solubility and catalyze the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of H_2O_2 to produce blue colored oxidized form of TMB (Okuda et al. 1996). Further, Li et al. employed the peroxidase mimicking activity of carboxyfullerenes for developing a colorimetric sensor of glucose. It was found that the carboxyfullerenes had better affinity towards the substrate (TMB) than natural enzyme [Horseradish peroxidase (HRP)]. The obtained peroxidase mimicking activity was found to be related with the electronic structure of the fullerenes. The developed sensor could efficiently detect glucose in a range from 1.0–40 μM in human serum and in other relevant buffers (Li et al. 2013).

3.2.2 Carbon Nanotubes

Carbon nanotubes (CNTs) are another class of carbon allotropes with certain unique characteristics such as high strength, durability, high electrical and ther-mal conductivity and chemically modifiable structure (Tans et al. 1997). They are cylindrical in shape and described as rolled cylinders of graphene sheets. Depending upon the layers of graphene, CNTs can be classified into two sub-groups (1) Single wall carbon nanotubes (SWCNTs) having size or diameter ranging from 0.4 nm to 2.5 nm, and (2) Multi wall carbon nanotubes (MWCNTs) having diameter from

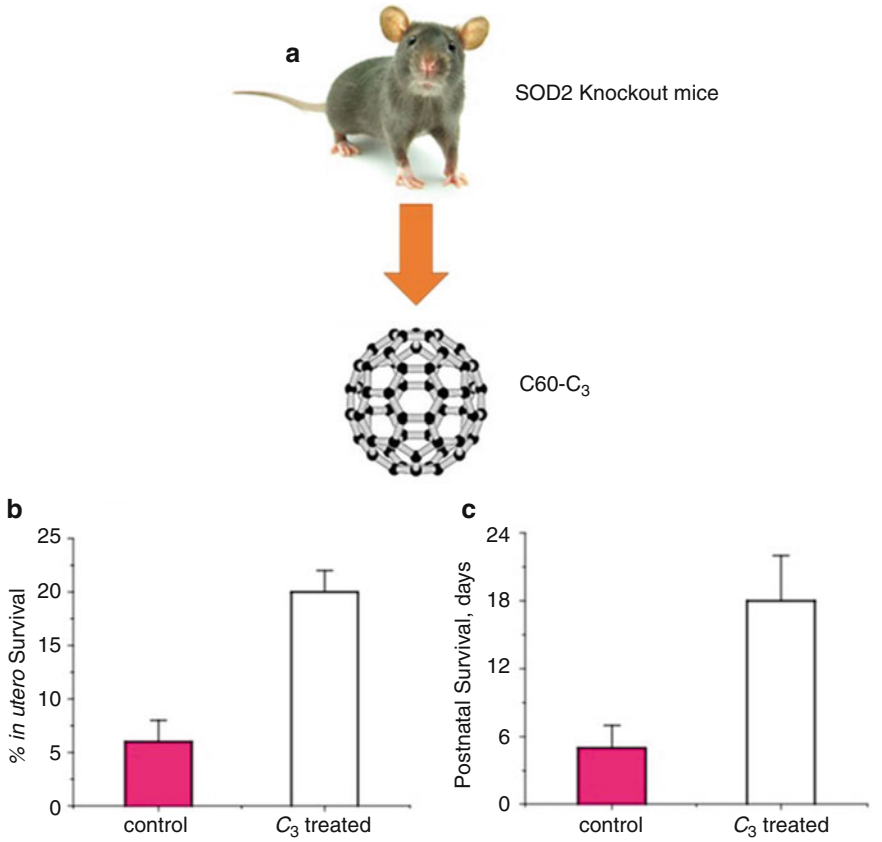

Fig. 3.2 (**a**) Figure showing the SOD2 knockout mice which was treated with C60-C_3. (**b**) % in utero survival of Sod2−/− pups born to Sod2+/− parents. C60-C_3 was given in drinking water to the pregnant dams (at 14–15th day of pregnancy). Dilute red food coloring was given to the control groups. The percentage survival of Sod2−/− pups, was found to be 6 ± 2% in control group, whereas survival percentage was found to be 20% ± 2 in C60-C_3-treated dams. (**c**) Survival of Sod2−/− pups (in days) which were treated with color-matched food coloring or C60-C_3 daily by subcutaneous injection. Reprinted with the permission from Ref. Ali et al. (2004) copyright © Elsevier

few nm to 100 nm. The interaction between layers of graphene in MWCNTs is through Van der Walls forces (Li and Chou 2003; Zhbanov et al. 2010). CNTs possess excellent properties however, their biological application is limited due to their poor stability in aqueous medium (Patel et al. 2019). Various methods are developed to improve their aqueous stability and thus biocompatibility. Among them, functionalization of CNTs by carboxylation of sidewall carbon atoms and subsequent modification by esterification or amination has shown excellent results (Sun et al. 2002; Hwang et al. 2013). Additionally, several polymers (Balasubramanian and Burghard 2005), biomolecules (Huang et al. 2002) and metals

(Lordi et al. 2001) are also grafted on the carboxylated surface of the CNTs which improved the solubility. However, biocompatibility remains an issue to be solved. CNTs have been explored to exhibit various enzymatic activities, and the subsequent section will briefly summarize the major reports in this direction.

3.2.2.1 Carbon Nanotubes as Peroxidase Mimic

SWCNTs are well reported for exhibiting biological peroxidase enzyme mimetic activities by Song et al. They reported that SWCNTs can catalyze the oxidation of substrate, TMB to produce the blue color in presence of H_2O_2 (Song et al. 2010a). Subsequently, Cui et al. studied the enzyme mimetic behavior of SWCNTs in presence of iron oxide nanoparticles (Fe_2O_3 NPs). Helical carbon nanotubes containing Fe_2O_3 NPs were synthesized. Surprisingly, peroxidase mimicking activity of SWCNTs was found to be dependent on the Fe concentration in the helix. However, SWCNTs showed much higher enzymatic activity as compared to MWCNTs even with very low amount of Fe (Cui et al. 2011). Song et al. studied the effect of silica coating on MWCNTs on the enzyme mimetic abilities of MWCNTs. Carboxyl modified CNTs were synthesized and coated with silica NPs. Silica decorated MWCNTs showed enhancement in the peroxidase mimetic activity of MWCNTs decorated with silica nanoparticles (SiNPs) (Song et al. 2010b).

3.2.2.2 Carbon Nanotubes as SOD Mimics

CNTs are also reported to possess natural SOD mimetic activity. Lucente et al. have studied the superoxide radical scavenging activity of SWCNTs after modification with a phenolic antioxidant (butylated hydroxyl-toluene (BHT)). Anchoring groups impart SOD-mimetic activity to the SWCNTs originally possessing limited aqueous solubility (SWCNT-1). To overcome this dilemma, wrapping of CNTs was attempted using polymer, pluronic, (SWCNT-2) and carboxylation (SWCNT-3). Subsequently, SWCNT-3 was further modified with polyethylene glycol (PEG) (SWCNT-4), which showed improved water solubility. The radical scavenging activity of these CNTs was compared with Trolox (a derivative of vitamin E) and results revealed that SWCNT-4 (without BHT modification) had greater enzymatic activity (almost 40 folds higher) towards radical scavenging than Trolox (Lucente-Schultz et al. 2009).

3.2.3 Graphene and Its derivatives

Graphene is a crystalline allotropic form of carbon with two dimensional properties. It shows characteristics of having few layered sheets of sp_2-hybridized carbon atoms, which are densely packed in a hexagonal lattice. A high specific surface area makes graphene a good candidate for aromatic drug loading and other catalytic activities (Cooper et al. 2012). Similar to other allotropes forms of carbon, pristine graphene also has solubility issues. Therefore, several derivatives of graphene have been developed including graphene oxide (GO), few layered graphene oxide (FLGO), reduced graphene oxide (rGO), and chemically changed graphene (CCG) (Patel et al.

2019). Recently, graphene and its derivatives have received significant attention due to their excellent catalytic activities.

3.2.3.1 Graphene Derivatives as Peroxidase Mimic

As discussed above, pristine graphene has limited solubility in water, therefore, several derivatives of graphene are developed and also studied for their biological enzyme mimetic activities. In a report by Song et al. the carboxyl-modified graphene oxide (GO-COOH) could show peroxidase enzyme mimetic activity (Song et al. 2010c) with a better affinity toward TMB than HRP. Graphene derivatives along with other carbon-based NPs generally composed of various oxygenated functional groups or moieties (hydroxyl, carboxyl, ketone, and epoxide etc.), which are the basis of their enzyme mimetic activities. So, to understand the role of these functional groups in the enzyme mimetic activity of graphene derivatives, graphene quantum dots were studied and the results revealed that carboxyl group acts as substrate binding site during the reaction and ketone group was found to be present at the active site, whereas hydroxyl group showed inhibitory role during the reaction (Song et al. 2010a). As hydroxyl group was found to be responsible for peroxidase mimetic activity of graphene quantum dots (GQDs), authors further studied the antibacterial activity in presence of H_2O_2. The results showed that the growth of bacteria (gram positive as well as gram negative bacteria) was inhibited by GQDs. The therapeutic effects was also studied in an injured mouse model and the obtained data suggested that GQDs have excellent antibacterial activity (Sun et al. 2014). The solubility of GOs was enhanced by coating polymers on the surface. Chitosan coated GO resulted in improved stability, solubility, and peroxidase mimicking activity (Wang et al. 2014a). Further, graphene decorated with other derivatives were also investigated for enzyme-mimetic activities. In this quest, Guo et al. developed hemin-rGO conjugate by decorating the hemin group on rGO. Here, hemin-rGO could catalyze the peroxidase reaction (Fig. 3.3) and followed the ping-pong mechanism (Guo et al. 2011). Au decorated GO and rGO are also studied in detail to understand the synergistic effect of the individual components (Liu et al. 2012b) (Table 3.2).

3.3 Metal-Based Nanozymes

Metal-based NPs are reported to display several applications in the area of nanomedicine because of their high biocompatibility and tunable properties (Rout et al. 2018). Metal-based nanozymes are also reported to exhibit a variety of biological enzymatic activities such as catalase, SOD, peroxidase, oxidase, phosphatase etc. (Kajita et al. 2007; Borghei et al. 2018; Wu et al. 2018; Su et al. 2015). Metallic nanozymes can be broadly divided into two types: Type I - NPs consisting of metallic core and assembled monolayer at the surface. These nanomaterials show enzymatic activities due to the monolayer molecules and not due to the core. Type II-NPs showing the enzymatic activities imparted by the metallic component. Metal nanoclusters have also been reported to as a potential candidate to function as a template for the self-assembly of many organic molecules (Pasquato et al. 2005).

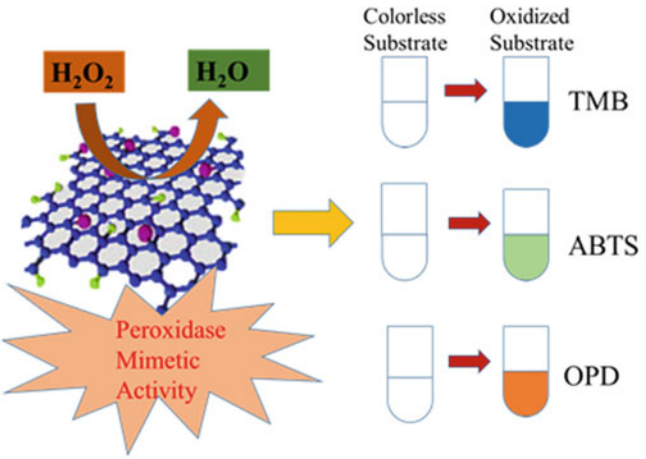

Fig. 3.3 Schematic presentation showing the peroxidase mimetic activity by hemin-rGO (hemin decorated reduced graphene oxide) conjugate. Nanozymes can oxidize various substrates (TMB, 2,2-azinobis (3-ethylbenzothizoline-6-sulfonic acid) (ABTS) and o-phenylenediamine dihydrochloride (OPD) in presence of H_2O_2 which results in blue, green and orange color, respectively

Among them, AuNPs are the most studied metal-based nanozyme, therefore, an elaborated discussion has been provided in the following section (Comotti et al. 2004, 2006; Luo et al. 2010).

3.3.1 Gold Nanoparticles

Au is one of the most studied metals with many attractive properties such as malleability, ductility and brightness. In addition, since Au is least reactive, it is considered as non-toxic to mammalian cells, therefore considered as one of the attractive agents for realizing biomedical applications. AuNPs are shown to display the catalytic activities of several biological enzymes.

3.3.1.1 Gold-Based Nanozymes as Peroxidase Mimic

Jv et al. have reported the peroxidase mimetic activity of positively charged AuNPs (Jv et al. 2010). Positively charged AuNPs were found to exhibit higher activity as compared to negatively charged AuNPs. As it was reported, purines have more adsorption on the surface of metals as compared to pyrimidines (Jang 2002). Wu et al. investigated the effect of purine derivatives for capping the surface of AuNPs and compared poly-purine and poly-pyrimidine capping over AuNPs and studied their peroxidase mimetic activity. Results revealed that there was significant enhancement in peroxidase mimetic activity in AuNPs capped with poly-purine than poly-pyrimidine derivatives. In case of 2, 6-diaminopurine (DAP) modified AuNPs the enhanced peroxidase mimetic activity was due to ferric ions present in

Table 3.2 Table summarizing various carbon based nanomaterials acting as nanozymes

Sr. No.	Type of carbon based nanomaterial	Functional group	Enzyme mimetic activity	References
1	Fullerenes (C60–1)	–	Nuclease mimetic activity	Tokuyama et al. (1993)
2	Fullerenes (Fullerene-1-ol and Fullerene-2-ol)	–	SOD mimetic activity	Dugan et al. (1996)
3	Fullerene (C60-C_3)	–	SOD mimetic activity	Chen et al. (2011)
4	Fullerene (C60-dimalonic acid)	–	Peroxidase mimetic activity	Okuda et al. (1996)
5	Fullerene (C62 $(COOH)_4$)	–	Peroxidase mimetic activity	Okuda et al. (1996)
6	CNTs	–	Peroxidase mimetic activity	Song et al. (2010c)
7	CNTs	Iron oxide NPs	Enhanced peroxidase mimetic activity	Cui et al. (2011)
8	CNTs	Silica NPs	Enhanced peroxidase mimetic activity	Song et al. (2010b)
9	CNTs	Butylated hydroxytoluene (BHT)	SOD mimetic activity	Lucente-Schultz et al. (2009)
10	Graphene derivatives	Carboxyl group	Peroxidase mimetic activity	Song et al. (2010c)
11	Graphene oxide	Chitosan	Enhanced peroxidase mimetic activity	Wang et al. (2014b)
12	Reduced graphene oxide	Gold	Enhanced peroxidase mimetic activity	Liu et al. (2012a)

the DAP (Wu et al. 2018). Subsequently, Jiang et al. reported that the chitosan-based AuNPs act as a good peroxidase mimic (Jiang et al. 2017). The enzymatic activity was measured by following the TMB oxidation and color change and found that the activity of chitosan coated AuNPs was higher than natural enzyme (HRP) over a wide range of temperatures (10–90 °C). Utilizing the peroxidase enzyme mimetic activity of AuNPs, Li et al. reported a novel colorimetric method for the detection of cysteine (Li et al. 2016a). It was found that in acetate buffer (pH 4.0), presence of cysteine results in the aggregation of AuNPs, which results in diminished peroxidase mimetic activity. Chai et al. developed a technique for the detection of lead using glutathione (GSH) functionalized peroxidase mimetic AuNPs (Chai et al. 2010). Aggregation of GSH-AuNPs was observed in presence of lead leading to the change in color of the suspension from pink to blue. The limit of detection was found to be 100 nM. AuNPs modified with cysteamine were also investigated for the detection of sulfate ions on the basis of inhibition of peroxidase mimetic activity (Zhao et al.

2015). Results showed that AuNPs coated with cysteamine undergo aggregated in presence of sulfate ions, due to electrostatic interactions and hydrogen bonding, which diminished the peroxidase mimetic activity.

It is well known that pH plays an important role in the catalytic activity of biological enzymes. Therefore, AuNPs are also studied for the efficiency of peroxidase mimetic activity in varying pH. It has been observed that the peroxidase mimetic activity of AuNPs was best at acidic pH (around 4.0 pH) but at physiological or alkaline pH, the activity gets compromised. Recently, it was shown that the peroxidase mimetic activity of AuNPs could be tuned to neutral pH by including some co-catalysts. In this context, Hu et al. studied the effect of heparin for the improvement of enzymatic activity of AuNPs at neutral pH (Hu et al. 2018). In the presence of heparin, the peroxidase mimetic activity of AuNPs was increased by 25-folds. Heparin addition not only enhance the peroxidase mimetic activity but also prevented AuNPs from catalyst deactivation. The effect of ATP on enzymatic activity of AuNPs was studied by Shah et al. and it was found that the peroxidase mimetic activity could be increased by the addition of ATP and ADP. Addition of similar anions such as sulphate, phosphate and carbonate anions did not have any impact on the enzymatic activity. Further, it was shown that the enzymatic activity was diminished after addition of ascorbic acid in the system even after addition of ATP and ADP showing that the enzymatic activity was due to the generation of hydroxyl radicals from H_2O_2 (Shah et al. 2015).

3.3.1.2 Gold-Based Nanozymes Exhibiting Other Enzyme Mimetic Activities

Apart from peroxidase, Au based nanozymes have been also investigated for oxidase, catalase, glucose oxidase (GOx) and SOD enzyme mimetic activities. Cao et al. have developed a colorimetric technique for the detection of melamine (MA) by exploiting the oxidase mimetic activity of HRP-modified Au nanoclusters. It was found that mercury (Hg^{2+}) could suppress the oxidase mimetic activity of Au nanoclusters due to the metallophillic interaction of Hg and Au. Whereas the oxidase like activity was found to be maintained in MA, thereby aiding in restoration of oxidase-mimetic activity because MA has better affinity towards Hg and it binds with Hg and did not allow its interaction with AuNPs. Hence colorimetric assay was developed for quantitation of MA (Cao et al. 2016). Borghei et al. studied the interaction of miRNA/DNA and cysteine on the surface of Au nanoclusters and found the inhibition of oxidase mimetic activity of the nanoclusters (Borghei et al. 2018).

Huang and Fan developed a novel technique which showed self-limiting growth of AuNPs on the basis of their GOx mimicking activity (Luo et al. 2010). They showed the oxidation of glucose catalyzed by AuNPs which produced H_2O_2 that induced the seed mediated growth of AuNPs in presence of chloroauric acid ($HAuCl_4$). This induced growth was regulated by internal factors. They demonstrated that the enzymatic activity, shape and size of NPs collectively controlled in a self-limiting manner. Zhang et al. have reported that bare AuNPs exhibit GOx mimetic activity whereas in presence of protectors the enzymatic activity could

be inhibited (Zhang et al. 2018). It has been reported that the accumulation of heavy metals on the surface of AuNPs can increase the enzyme mimetic activities. In this context, the catalase mimetic activity of AuNPs in presence of heavy metal ions has been studied by several groups. Among them, He et al. have demonstrated that AuNPs can efficiently decompose H_2O_2 with the production of hydroxyl radicals at lower pH and O_2 at higher pH. Further, it was also demonstrated that AuNPs could catalyze the degradation of superoxide radicals, thus mimicking the activity of SOD enzyme too (He et al. 2013).

3.3.2 Platinum Nanoparticles

Platinum (Pt) is also considered as a noble metal as it has very less reactivity. Platinum nanoparticles (PtNPs) have been investigated for many enzyme mimetic activities such as SOD, catalase, peroxidase and oxidase.

3.3.2.1 Platinum Nanoparticles as Enzyme Mimics

Kajita et al. have reported SOD and catalase enzyme mimetic activities in PtNPs. In an attempt, bimetallic NPs of Au and Pt was prepared which could result in the free radical scavenging similar to the natural SOD and catalase enzymes (Kajita et al. 2007). Shibuya et al. have investigated the antioxidant activity of PtNPs in animal models. Here, a combination of Pt and palladium nanoparticles (PdNPs) was synthesized to investigate the ROS scavenging ability of these NPs. Results showed that the PtNPs could mimic the activity of SOD as well as catalase enzyme, whereas PdNPs showed limited catalytic activity (Shibuya et al. 2014). Fan et al. demonstrated the catalase and peroxidase mimetic activity of PtNPs using ferritin-PtNPs. It was also observed that these biomimetic activities were dependent on the temperature and pH. Maximum catalase mimetic activity was observed at alkaline pH and higher temperature whereas peroxidase mimetic activity was found to be maximum at lower pH (acidic) (Fan et al. 2011). He et al. demonstrated that Pt nanodots coated over Au nanorods exhibit oxidase, peroxidase and catalase mimetic activities. Using these nanozymatic activities, authors developed an enzyme linked immunosorbent assay (ELISA) for detection of mouse interleukin-2 (IL-2) (He et al. 2011). Further Liu et al. showed that platinum nanodots coated over Au nanorods could show peroxidase and ascorbate oxidase enzyme mimetic activities (Liu et al. 2012b). Deng et al. have studied the effect of capping on the enzyme mimetic activity of PtNPs and observed that chitosan capping increases the stability as well as the enhancement of the oxidase mimetic activity (Deng et al. 2017).

3.3.3 Silver Nanoparticles

Silver (Ag) NPs are known for exhibiting inherent antimicrobial properties. Pollution, dynamic environmental conditions and mutations results in development of various drug resistant viruses and bacteria. AgNPs have been investigated very effective in controlling the growth of such infectious bacteria (Jones et al. 2004;

Silver and Phung 1996). Apart from having antimicrobial potential, AgNPs have been investigated for various enzyme mimetic activities which have been discussed in the following section.

3.3.3.1 Silver Nanoparticles as Enzyme Mimics

AgNPs have been reported to exhibit oxidase, peroxidase, SOD and catalase enzyme mimetic activities (Tyagi et al. 2016; McKeating et al. 2013; Wang et al. 2014b). Wang et al. showed that AgNPs show oxidase mimetic activity in presence of mercury (Wang et al. 2014a). They synthesized citrate capped AgNPs and found that mercury(II) ion enabled AgNPs were able to catalyze the oxidation of substrate TMB. Further they demonstrate that the oxidase mimetic activity of AgNPs is dependent on the concentration of mercury ions and it was specific for mercury (II) ions only. In another study, peroxidase mimetic activity of AgNPs was reported by Jiang et al. and it was found that chitosan coated AgNPs have the potential to catalyze the oxidation of substrates (TMB and OPD) in presence of H_2O_2 and produce blue and red colors, respectively. The prepared NPs were resistant to pH and temperature and therefore could be used for catalytic reactions under harsh conditions as compared to natural HRP enzyme. Their electron paramagnetic resonance (ESR) studies showed that Ch-AgNPs catalyze the decomposition of H_2O_2 into hydroxyl radicals (Jiang et al. 2012). McKeating et al. also demonstrated the peroxidase mimetic activity of AgNPs. Utilizing peroxidase mimetic activity of AgNPs along with Surface Enhanced Resonance Raman Scattering (SERRS), a unique method of spectroscopic analysis was developed for H_2O_2 detection with a limit of 100 nM (McKeating et al. 2013). Yesmurzayeva et al. synthesized AgNPs in presence of poly (N-vinyl pyrrolidone) (PVP). They supported the polymer coated AgNPs on the surface of zinc oxide (ZnO) and then they analyzed the catalytic activities of NPs. They followed the decomposition of H_2O_2 at optimal conditions and found that H_2O_2 decomposition was dependent on the molecular weight of PVP, temperature, concentration of substrate and amount of catalyst. Hence they showed catalase like behavior of the polymer coated AgNPs (Yesmurzayeva et al. 2015). Tyagi et al. studied the activity of AgNPs against UV-B induced DNA damage (Tyagi et al. 2016). They checked the efficacy of Ag, Titanium oxide (TiO_2) and Zinc oxide (ZnO) NPs in preventing the radiation (UV-B) induced damage in HaCaT cells. They observed that AgNPs were able in protecting HaCaT cells against oxidative DNA damage (caused by UV-B radiation) by improving the SOD and catalase mimetic activity.

3.3.4 Other Metallic Nanoparticles as Enzyme Mimics

Apart from above mentioned metallic NPs there are some other metallic NPs are present and are reported for their enzyme mimetic activities, which are discussed in the following section.

3.3.4.1 Palladium Nanoparticles as Enzyme Mimics

Palladium (Pd) is a transition metal with unique electronic, mechanical and catalytic properties. Such as oxidation (Karimi et al. 2006), and hydrogenation (Zhao and Crooks 1999) etc. Use of Pd in medicines is common such as in dental implants (Woodward 2012), prostate cancer (Blasko et al. 2000), and choroidal melanoma (Finger et al. 2009) etc. Apart from these activities, PdNPs are also reported for exhibiting enzyme mimetic activities. Wang et al. investigated the interaction of nickel (Ni) with PdNPs and observed that Ni-Pd hollow NPs exhibit three different enzymatic activities: oxidase, catalase and peroxidase. On the basis of peroxidase mimetic activity of Ni-PdNPs, a method of glucose biosensor with high efficiency, sensitivity and specificity was developed (Wang et al. 2016). Methods are also reported which can enhance the peroxidase activity of PdNPs. In this context, Li et al. developed PdNPs coated with magnetic graphene nanosheets and followed the effect of coating on the enzymatic activity. It was observed that graphene nanosheets coating could significantly increase the peroxidase mimetic activity of the NPs (Li et al. 2016b).

In another study by Rastogi et al., effect of GK (gum kondagogu), a biopolymer, was investigated towards stabilizing the NPs and improving the enzyme mimetic activities. GK reduced PdNPs was prepared and the peroxidase mimetic activity was studied following the oxidation of TMB in presence of H_2O_2. Results suggested that GK-PdNPs were stabilized by coating and also exhibited intrinsic peroxidase mimetic activity in varying pH and temperature conditions (Rastogi et al. 2017). Moglianetti et al. studied the effect of capping on the antioxidant activity by utilizing stable citrate-capped PdNPs with size range from 4–8 nm. The two sizes (4 and 8 nm) of PdNPs was synthesized and cellular uptake was studied by Inductively Coupled Plasma Atomic Emission spectroscopy (ICP-AES) and Sputtering-Enabled Intracellular X-Ray Photoelectron Spectroscopy (SEI-XPS). Results showed that citrate capped PdNPs have the potential to mimic three different enzymes activity: SOD, catalase and peroxidase, thus can efficiently scavenge ROS from cytoplasm (Moglianetti et al. 2020).

3.3.4.2 Iridium Nanoparticles as Enzyme Mimics

Iridium nanoparticles (IrNPs) are unique transition metal NPs which belongs to platinum group. Su et al. studied the effect of Polyvinylpyrrolidone (PVP) coating on the IrNPs. The synthesis of PVP stabilized IrNPs was performed by alcoholic reduction method and the so developed NPs could show catalase and peroxidase enzyme mimetic activities. Further, they also found that the catalase mimetic activity was higher in basic and neutral pH whereas peroxidase mimetic activity was best in acidic pH. The catalytic activities of PVP-IrNPs were found to be comparable with bare PtNPs when investigated for exhibiting antioxidant activity of IrNPs in A549 lung cancer cell line (Su et al. 2015).

3.3.4.3 Rhodium Nanoparticles as Enzyme Mimics

Rhodium(Rh) is a noble metal having specific catalytical applications. Choleva et al. firstly reported peroxidase mimetic activity of RhNPs. They showed that RhNps

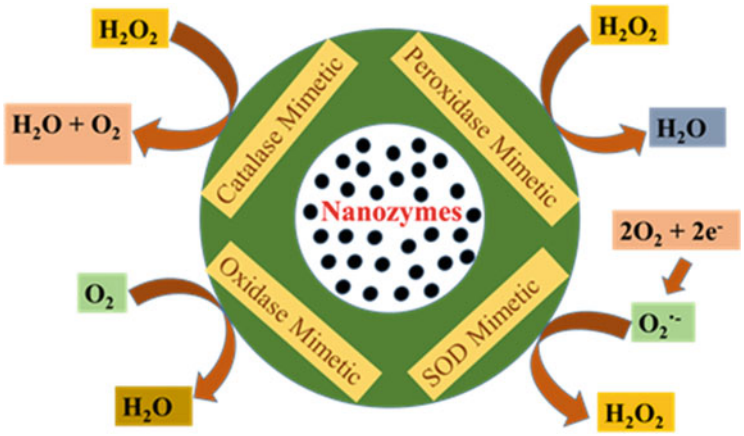

Fig. 3.4 Figure shows different types of enzymatic activities exhibited by nanomaterials acting like enzymes (nanozymes)

have the potential to catalyze the oxidation of substrate TMB in presence of H_2O_2. They clearly showed that the RhNPs were following Ping Pong mechanism which is attributed to transfer of electrons between substrate TMB and hydroxyl radicals (generated by decomposition of H_2O_2 catalyzed by NPs). RhNPs offer several advantages over other NPs such as higher affinity towards peroxidase substrate, high reaction velocity, easy and facile synthesis, and lower limit of detection (Choleva et al. 2018) (Fig. 3.4).

3.3.4.4 Copper Nanoparticles as Enzyme Mimics

Copper nanoparticles (CuNPs) are well-known for exhibiting unique surface plasmon resonance and antimicrobial and antifungal applications (Ramyadevi et al. 2012). CuNPs are also reported to be used as biosensors and exhibit enzyme mimetic activities. Xu et al. have shown the peroxidase enzyme mimetic activity in copper nanoclusters and used them to develop a specific and sensitive chemiluminescence (CL) sensor for the detection of cholesterol. Copper nanoclusters can efficiently catalyze the CL reaction between H_2O_2 and luminol. Here, H_2O_2 is produced due to the oxidation of cholesterol in presence of cholesterol oxidase. The developed sensor was applied for the detection of cholesterol in milk powder and human serum (Xu et al. 2016). Further, Yan et al. have developed a method for the detection of xanthine in human serum utilizing the peroxidase mimetic activity of copper nanoclusters (Yan et al. 2017).

3.4 Metal-Oxide Based Nanozymes

Since last few years metal oxide NPs have gained tremendous attraction of scientists due to their enzyme mimetic activities. In 2007, Fe_2O_3 NPs were first discovered for exhibiting intrinsic peroxidase enzyme mimetic activity (Gao et al. 2007). Subsequently, a wide range of metal oxides were investigated for possessing enzyme mimicking activity, among them cerium oxide NPs (Nanoceria), copper oxide NPs (CuO), TiO_2 etc. are some of the most studied NPs. section.

3.4.1 Cerium Oxide Nanoparticles (Nanoceria)

Cerium (Ce) is one of the rare earth metals belonging to lanthanide series, however, unlike other rare earth elements, cerium can exist in two possible oxidation states (3+ and 4+). The two forms of nanoceria i.e. nanoceria (3+) (partially reduced form) and nanoceria (4+) (fully oxidized form) have redox switching ability that is considered as the key factor behind their enzyme mimetic activities such as SOD, catalase, oxidase, peroxidase, phosphatase, and phosphotriesterase etc. (Yadav et al. 2019).

3.4.1.1 Nanoceria as SOD Mimic

SOD enzyme is one of the major antioxidant biocatalyst involved in the balancing of the redox status of the mammalian cells. The first report of nanoceria as SOD mimetic was published by Korsvik et al. in 2007 (Korsvik et al. 2007). Nanoceria bearing higher 3+ ions on the lattice surface were found to exhibit SOD enzyme mimetic activity that was found to be dependent on the 3+/4+ ratio of the "Ce" atoms present on the surface of the NPs. Dhall et al. later reported that nanoceria also have surface regeneration ability, i.e. nanoceria could regenerate the 3+ oxidation state atoms, leading to the enhanced enzyme mimicking ability (Dhall and Self 2018). Subsequently, Li et al. studied the interaction of nanoceria with natural Cu-Zn-based SOD enzyme and demonstrated that the overall SOD activity of nanoceria gets improved by combining Cu-Zn-based SOD or an electron donor molecule [Ru(dcbpy)2(NCS)2] (Li et al. 2015; Wu et al. 2014). The effect of surface coating over the enzyme mimetic activity of nanoceria has also been studied in detail. Nanoceria has been functionalized with bovine serum albumin (BSA), dextran, and polyethylene glycol (PEG) where it was found that PEG and BSA coating did not alter their SOD mimetic activity. Additionally, nanoceria was encapsulated in Poly (lactic-co-glycolic acid) (PLGA) NPs and results suggested that nanoceria was able to maintain its SOD mimetic activity when released from PLGA NPs (Singh et al. 2012). Singh et al. have studied the impact of biologically relevant buffers on the enzyme mimetic activity of nanoceria. The study reported that the interaction of phosphate, buffer with nanoceria leads to the loss of SOD mimetic activity whereas other corresponding ions such as sulfate and carbonate did not alter the the activity (Singh et al. 2011).

3.4.1.2 Nanoceria as Catalase Mimic

Nanoceria containing surface atoms with high 4+ oxidation state is reported to exhibit catalase mimetic activity. Pirmohamed et al. were first to report the catalase mimetic activity in nanoceria by following the degradation of H_2O_2 by various methods, including UV-Vis spectroscopy (observing the decrease in absorbance of H_2O_2 at 240 nm), analyzing released molecular oxygen by oxygen probe, and amplex red assay (Pirmohamed et al. 2010). Singh et al. studied the impact of phosphate buffer, pH and cell culture media on the catalase mimetic activity and stability of nanoceria. The results revealed that there was no effect on the catalase mimetic activity of nanoceria in presence of these factors which suggests that these biological buffers do not interact with the active site of nanoceria (Singh and Singh 2015). In vitro studies performed by Singh et al. proved that nanoceria could protect the human liver cell line (WRL-68), stressed from excess of H_2O_2, produced by inhibiting the natural catalase enzyme by 3-Amino-1,2,4- Triazole (3-AT) (Singh and Singh 2019).

3.4.1.3 Nanoceria as Oxidase Mimic

The oxidase mimetic activity of nanoceria was first reported by Asati et al. at acidic pH. It was found that at acidic pH nanoceria could to catalyze the oxidation of substrates such as TMB and ABTS and produced blue and green color solution, respectively in presence of oxygen (Asati et al. 2009). Subsequently, in a study by Ma et al. the effect of capping on the catalytic ability of nanoceria was reported. It was demonstrated that nanoceria capped with fluoride ions (F^-) could enhance the the oxidase mimetic activity. Fluoride as a capping agent was selected on its binding affinity, as fluoride is Lewis base and nanoceria is acidic in nature therefore, a strong adsorption of the fluoride on the surface of nanoceria was observed, which lead to the 100 folds increase in oxidase mimetic activity (Ma et al. 2018).

3.4.1.4 Nanoceria as Peroxidase Mimic

Heckert et al. in 2008, first reported that nanoceria could produce hydroxyl radicals by following the Fenton-like reaction (Heckert et al. 2008). Subsequently, Asati et al. demonstrated that nanoceria could exhibit peroxidase mimetic activity (Asati et al. 2011). It was observed that nanoceria can catalyze the oxidation of TMB (substrate) in presence of H_2O_2 in a pH dependent manner (between pH 2–6) (Jiao et al. 2012). Vinothkumar et al. studied the $CePO_4$-CeO_2 nanorods interaction and found that the composite could show peroxidase mimetic activity. Subsequently, this composite was used for the detection of glucose and H_2O_2 at concentration of 150 μM with a limit of detection (LOD) of 2.9 and 4.1 μM, respectively. This observation was linked to the redox switching mechanism shifting between 3+ and 4 + oxidation state of nanoceria among the surface atoms of "Ce" of $CePO_4$-CeO_2. (Vinothkumar et al. 2018).

3.4.1.5 Nanoceria as Other Enzyme Mimic

Phosphatases are the group of enzymes that can degrade phosphoric acid and release inorganic phosphate as end product. Kuchma et al. firstly investigated the phosphatase mimetic activity of nanoceria dependent on the number of Ce^{3+} on the lattice

surface by following the degradation of p-nitrophenyl phosphate (pNPP) at 405 nm. It was demonstrated that nanoceria can interact with the phosphoester bond of p-nitrophenyl phosphate and release the inorganic phosphate, which can be quantitatively measured. Study revealed that at acidic, neutral, and alkaline pH could alter the reaction with ~1.7-fold enhanced activity at pH 4 than pH 7, and ~ tenfold faster than pH 10. The effect of binding of polyoxometalates (keggin ions) on the catalytic activity of nanoceria has also been studied where phosphatase mimetic activity was selectively inhibited by kegging ions whereas catalase mimetic activity was unaffected. This investigation suggested that the active sites, similar to natural enzymes, are present on nanoceria surface, and are different for catalase and phosphatase mimetic activity (Kuchma et al. 2010).

Phosphotriesters or organophosphates are toxic chemical compounds generally used in pesticides for crop protection against harmful insects. The enzymes responsible for the detoxification of organophosphates in human body are known as phosphotriesterases. Nanoceria has also been discovered to exhibit phosphotriesterase enzyme mimetic activity (Vernekar et al. 2016). In this context, Verneker et al. have demonstrated that vacancy engineered nanoceria could exhibit phosphotriesterase enzyme mimetic activity by following the formation of p-nitrophenolate (end product of the reaction) and measuring the absorbance at 401 nm. Incubation of nanoceria with paraoxon (phosphotriester) did not cause any hydrolysis, whereas addition of histidine to the reaction lead to the low phosphotriesterase enzyme mimetic activity. Subsequently, replacement of histidine with *N*-methylmorpholine (NMM) lead to the significant increase in hydrolysis. Conversely, use of other metal oxides such as TiO_2 and ZnO did not show any phosphotriesterase enzyme mimetic activity.

3.4.2 Iron Oxide Nanoparticles

Iron oxide nanoparticles (Fe_3O_4 and Fe_2O_3) were the first metal oxides discovered for their enzyme mimetic activity. They are extensively studied among metal oxides. IONPs exhibits peroxidase and catalase mimetic activities.

3.4.2.1 Iron Oxide Nanoparticles as Peroxidase Mimic

Gao et al. in 2007 reported that IONPs of different sizes were having peroxidase mimetic activity. They showed the oxidation of various substrate such as TMB, OPD and diazoaminobenzene (DAB), to give the products of blue, orange and brown colors respectively (Gao et al. 2007). Further they demonstrated that the Michaelis-Menton constant (Km) was very low, even lesser than the HRP. These results suggested that IONPs has much higher affinity towards substrate TMB than HRP. The peroxidase mimetic activity is reported in both Fe_2O_3 and Fe_3O_4 types of NPs. Vallabani et al. showed the impact of ATP on the peroxidase mimetic activity of Fe_3O_4 NPs. Results suggested that peroxidase mimetic activity of Fe_3O_4 NPs is pH dependent and the maximum activity was observed in acidic pH (see Fig. 3.5). However, significant peroxidase mimetic activity was observed at physiological pH

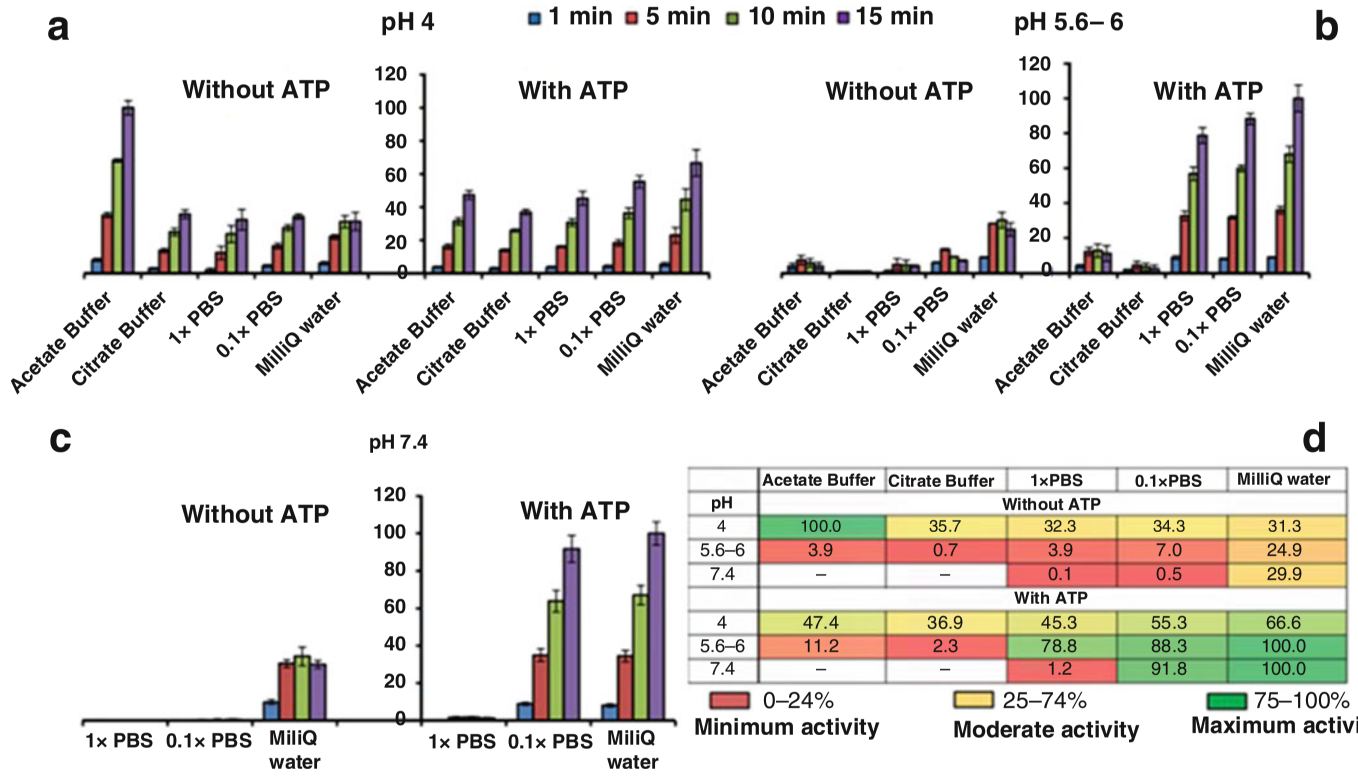

Fig. 3.5 Comparative analysis of peroxidase mimetic activity Fe_3O_4 nanoparticles at different pH conditions. Various buffers were used to compare the enzymatic activity of nanoparticles. (**a**) Peroxidase mimetic activity of Fe_3O_4 nanoparticles at pH 4 in absence and presence of ATP. (**b**) at pH 5.6–6 in absence and presence of ATP. (**c**) at pH 7.4 in absence and presence of ATP and (**d**) their heat map analysis. Reprinted with the permission from Ref. Vallabani et al. (2017) copyright © Elsevier

after including ATP in the reaction buffer. They showed that in presence of ATP the intrinsic peroxidase mimetic activity got enhanced and exists over a wide range of pH and temperature. They utilized ATP enhanced peroxidase mimetic activity of IONPs for the development of detection technique (single step) for glucose and the limit of detection was 50 μM (Vallabani et al. 2017). Ma et al. developed a novel technique to determine the level of reduced GSH on the basis of peroxidase mimetic activity (Ma et al. 2012).

3.4.2.2 Iron Oxide Nanoparticles as Catalase Mimic

Chen et al. reported the catalase mimetic activity of iron oxide nanoparticles. They showed that catalase mimetic activity of nanoparticles is pH dependent. To understand the interaction of iron oxide nanoparticles with H_2O_2 they studied the effect of iron oxide nanoparticles on human glioma U251 and they observed concentration dependent cytotoxicity. Their electron spin resonance spectroscopy results suggested that both Fe_3O_4 and Fe_2O_3 nanoparticles were catalyzing the degradation of H_2O_2 to produce hydroxyl radicals in lysosomes which was confirming the peroxidase mimetic activity of nanoparticles whereas in cytosol H_2O_2 was decomposed into H_2O and O_2 which showed the catalase mimetic activity of iron oxides (Chen et al. 2012a).

3.4.3 Other Metal Oxide Nanoparticles

Apart from iron and cerium oxides, many other metal oxides have been reported for their enzyme mimicking behavior such as CuO, TiO_2, vanadium oxides (VO), manganese oxides (MnO) etc. Depending on the shape and size, these NPs display different enzyme mimetic activities.

3.4.3.1 Other Metal Oxide Nanoparticles as Enzyme Mimics

VOx nanoparticles has been investigated for many enzyme mimetic activities. André et al. showed V_2O_5 nanowires as peroxidase mimic. They demonstrated that V_2O_5 NPs in presence of H_2O_2 can catalyze the oxidation of substrate TMB and ABTS at pH 4 and the activity was found to be concentration dependent (André et al. 2011). In another study Zhang et al. reported the peroxidase mimetic activity of VO_2 nanoplates (Zhang et al. 2015). They synthesized VO_2 nanoplates by hydrolysis of concentrated $VO(acac)_2$ solution. They evaluated the peroxidase like activity of nanoplates by following the oxidation of TMB and found them biomimetic catalyst.

CuO were identified as oxidase and peroxidase mimics. Hu et al. demonstrated that cupric oxide NPs act as oxidase mimic as they were able to catalyze the aerobic oxidation of cysteine to cysteine and resulted in the production of H_2O_2. They coupled this property with the peroxidase mimetic activity of CuO NPs and constructed a cascade reaction that includes the oxidation of cysteine into cysteine which results in production of H_2O_2 and that H_2O_2 catalyzed the oxidation of Terephthalic acid (TA) to generate a fluorescence change. On the basis of their technique they developed a fluorescent assay for the determination of cysteine with a

detection limit of 6.6 nM (Hu et al. 2017). Chen et al. identified the peroxidase mimetic activity of water soluble CuO and on that basis developed a glucose detection technique. (Chen et al. 2012b).

3.5 Other Biomedical Applications of Nanomaterials

Advancement of the characterization and imaging techniques promotes the use of nanomaterials as a medicine which opens a new research field named as a nanobiotechnology. Nanobiotechnolgy plays an important role in disease diagnosis, drug delivery, and implants. Unique chemical and physical property of nanomaterials allow them to applicable in diagnosis, bio sensing and bio imaging devices, drug delivery systems, and bone substitute implants. Nanomaterials like metal and metal oxide NPs and carbon-nanotubes have wide range of biological applications. Among these nanomaterials metal and metal oxide NPs are widely in use. NPs like nanoceria (Das et al. 2007), ZnO (Zhang et al. 2013), CuNPs (Goel et al. 2014), AgNPs (Burdușel et al. 2018) AuNPs (Cabuzu et al. 2015), IONPs (Magro et al. 2018) and so on, these particles have proven their biomedical application for various purposes through several studies. Metal oxide NPs are applicable for constructing different medical devices. One good example is application of IONPs for therapeutic and diagnostic purposes, in magnetic particle imaging, magnetic resonance imaging, ultrasonic technique, photoacoustic imaging, and magnetic particle hyperthermia as a contrast agent (Oh et al. 2006), photoacoustic imaging, and magnetic particle hyperthermia (Liu et al. 2016). Intrinsic fluorescence properties of ZnO nanowires have application in imaging of cancer cells (Hong et al. 2011). Titanium oxide (TiO_2) has a wide range of applications in bone-substituting materials and bone regeneration (Tan et al. 2012). Zirconium oxide has compatibility with hard tissue and has been used for dental implants (Özkurt and Kazazoğlu 2011; Ramos et al. 2017). Nanoceria have established themselves as potential anti-oxidants by showing their catalytic effect in the test tube, cell culture models and animal models of disease. Initially, nanoceria was used as a glass polishing, chemical or physical polishing agent or as fuel additives. In earlier 2006, groundbreaking study on nanoceria proved its potential biomedical applications in cell culture model (Tarnuzzer et al. 2005; Chen et al. 2006).

3.6 Conclusion and Future Directions

Over the recent years, extensive attention has been given to the nanomaterials exhibiting biological enzyme mimetic activities. The easy synthesis, high specificity to the substrate, easy purification, surface modification, and long term storage are some of the key characteristics of nanozymes which make them better alternative to the natural enzymes. Over the last two decades the attempts have been performed to improve the enzyme mimetic activities and stability of nanozymes in harsh environmental conditions. From the above discussion, it is evident that nanozymes are the

emerging inorganic catalysts which exhibit various enzymatic activities and can be used as surrogates of natural enzymes. Various free radicals are produced in our body as a part of metabolism but overproduction of these free radicals can harm our system. Nanozymes having SOD and catalase mimetic activities can be used against these free radicals but NPs in biological system face many issues such as corona (chemical or protein) formation, instability, loss of enzymatic activities, and cytotoxicity. To overcome these issues several attempts have been practiced which includes surface functionalization of NPs, doping and coating to small inorganic ions etc. Despite of attempting so much trials, still stability and interaction of NPs with bio-macromolecules is an issue. More efforts are required to understand the nano-bio interaction and corona formation in biological system. In-depth in vitro as well as in vivo studies are required in this direction to understand the mechanism of enzymatic action. These practices will improve the enzymatic activities, biocompatibility, stability and therapeutic effects of nanozymes.

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Biogenic Silver Nanoparticles: A Potent Therapeutic Agent

4

Shakil Ahmed Polash, Md. Monir Hossain, Tanushree Saha, and Satya Ranjan Sarker

Abstract

The evolution of bacterial resistance to antibiotics has become a real threat to the global public health. Bacteria alter their genome to survive in the antibiotic stressed condition which result in the development of antibiotic resistance mechanism. Infelicitous use and abuse of antibiotics are the main cause of the development of antibiotic resistance mechanism. Chemically synthesized antibiotics is hazardous for the environment, and bacteria can easily develop a resistance mechanism through modification of their genetic machinery. However, biogenic silver nanoparticles are synthesized using the biomass of plant extract. Unlike chemically synthesized antibiotics, the biogenic silver nanoparticles first bind with the bacterial membrane through noncovalent interactions before manifesting their antibacterial activity. Therefore, it is highly unlikely for the bacteria to develop resistance mechanism against biogenic silver (Ag) nanoparticles. Furthermore, the synthesis procedure of biogenic nanoparticle is ecofriendly, cost effective and they are highly biocompatible. On the other hand, biogenic Ag nanoparticles usually spread uniformly throughout the tissues. They also have anticancer potential and stimulate the apoptotic pathways of cancer cells. In this chapter, we have summarized the important synthesis pathways of biogenic Ag nanoparticles as well as their biomedical applications (i.e., antibacterial,

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antioxidant, anticancer, antifungal, antiparasitic, antidiabetic, and wound healing activities).

Keywords

Biogenic silver nanoparticles · Antibacterial activity · Anticancer activity · Apoptotic pathways

4.1 Introduction

Nowadays green chemistry has become one of the most sought after technologies for the development of non-hazardous and environment friendly chemical processes to synthesize nanoparticles using biodegradable and sustainable materials. The nanoparticles are in high demand because of their small size, and large surface-to-volume ratio. Furthermore, the nanoparticles also possess excellent optical, electrical, magnetic, and catalytic properties. Metallic nanoparticles have attracted wide attention among the nanomaterials, especially in the field of medical science and engineering, because of their unique nanometric properties as well as the possibility of modification and surface functionalization (Edmundson, Capeness, & Horsfall, 2014; Mody, Siwale, Singh, & Mody, 2010). Although the physical and chemical processes produce nanoparticles with high purity, the chemicals used in the synthesis are expensive, toxic, energy consuming, produce hazardous by-products and thus preventing their clinical and biomedical applications (Azizi, Namvar, Mahdavi, Ahmad, & Mohamad, 2013; Das, Nath, Chakdar, Gope, & Bhattacharjee, 2010; El-Rafie, El-Rafie, & Zahran, 2013; Faramarzi & Sadighi, 2013; Ghaffari-Moghaddam, Hadi-Dabanlou, Khajeh, Rakhshanipour, & Shameli, 2014; Prasad, Kambala, & Naidu, 2013; Vaidyanathan, Kalishwaralal, Gopalram, & Gurunathan, 2009). There is a growing need to build an environment friendly method for the synthesis of nanoparticles which does not use noxious chemicals. Recently, scientists have established a novel technology for the synthesis of nanoparticles using biomass of either microorganisms or plants. Biosynthesized nanoparticles are more ecofriendly and biocompatible in clinical applications when compared to their chemically synthesized counterparts.

Green synthesis of AgNPs is a very quick and cost-effective process that satisfies the research community's demand and at the same time avoids the likelihood of environmental risks (Rogach, 2000). Besides the use of plant extracts, microorganisms such as bacteria, fungi, and algae are reported to have inherent potential to produce metal nanoparticles either intra- or extra-cellularly and are considered as potential biofactors for the synthesis of nanoparticles (Narayanan & Sakthivel, 2010).

Silver is a transition metal possessing high electrical and thermal conductivity (Firdhouse & Lalitha, 2015). During the ancient period of life, it was used as coins,

tubes, liquids, foil, sutures, and colloids as lotions, unguents, and so on (Alexander, 2009). Upon understanding the medicinal properties of silver, silver-based compounds have been widely used as antimicrobial agents (Husen & Siddiqi, 2014). Silver-based nanoparticles are considered to have significant bacteriostatic as well as bactericidal effects against a wide range of pathogens (Firdhouse & Lalitha, 2015; Nam, Purushothaman, Rangasamy, & Song, 2016). Hence, they have been used for centuries to prevent and cure various diseases, most notably infectious diseases (Ahmed, Ahmad, Swami, & Ikram, 2016). Silver nanoparticles also possess anti-inflammatory, antiviral and antiplatelet activity (Shankar, Rai, Ahmad, & Sastry, 2005). Due to the physical and chemical properties of biogenic AgNPs, they can be used for multiple purposes including diagnosis of bacterial infection and their treatment (Fayaz et al., 2010; Sharma, Yngard, & Lin, 2009). For example, they are used frequently as antimicrobial agents (Habiboallah et al., 2014; Nambiar & Bhathena, 2010; Singh & Singh, 2014), as topical creams to prevent wound infections (Tian et al., 2007), and as anticancer agents (Kaur & Tikoo, 2013). Furthermore, silver nanoparticles are also used in sensor technology, biological leveling, and many other biomedical applications (Asharani, Low Kah Mun, Hande, & Valiyaveettil, 2008; Gomez-Romero, 2001; Li et al., 2011; Patil, Kokate, & Kolekar, 2012; Pollini et al., 2011; Qiu, Rieger, Gilbert, Jérôme, & Jérôme, 2004).

In this chapter, we shall discuss on the synthesis of biogenic AgNPs and their potential applications.

4.2 Synthesis of Silver Nanoparticles

4.2.1 Biological Synthesis of Silver Nanoparticles

The biological synthesis of silver nanoparticles is preferred over their chemical synthesis because the biological synthesis is cost effective and non-hazardous to the environment. The size, shape, and composition of nanoparticles can be controlled by tuning their extraordinary optical, electrical, magnetic, thermal, catalytic, chemical, and biological properties. The synthesis of inorganic nanoparticles including silver (Ag), gold (Au), graphene oxide (GO), zinc oxide (ZnO) nanoparticles (NPs) have been widely investigated as antibacterial agents due to their tremendous physical, chemical and biological properties. Among them, silver nanoparticles (AgNPs) have been broadly used in different purposes including pharmaceuticals and cosmetics and in the treatment of water due to their wide range of antibacterial propensity against a broad spectrum of bacteria. However, the chemically synthesized AgNPs are highly prone to aggregation and become unstable because of their easy oxidation. The AgNPs lose their antibacterial activity due to their extreme aggregation tendency, and the leakage of Ag^+ from chemically synthesized AgNPs brings about human as well as environmental hazard.

Recently, some research groups have reported the synthesis of AgNPs using plant extracts to overcome the limitations of chemical synthesis. The synthesis of AgNPs using plant extracts is easier, ecofriendly, more effective and cost effective. The

phytoconstituents have better reducing potential and the as-synthesized biogenic AgNPs are more stable (Mohamad, Arham, Jai, & Hadi, 2014). For example, the aqueous and ethanolic extracts of *Andrographis paniculata* stem were used to produce aqueous biogenic AgNPs (Aq-bAgNPs) and ethanolic biogenic AgNPs (Et-bAgNPs) respectively, and both the bAgNPs were ~25 nm in size (i.e., diameter) (Hossain et al., 2019). The leaf extracts of *Eugenia jambolana*, *Rhynchotechum ellipticum*, and Hesperidin (Gomathi et al., 2017; Hazarika, Phukan, Saikia, & Chetia, 2014; Stephen & Seethalakshmi, 2013) were used in the synthesis of bAgNPs indicating the presence of flavonoids, saponins, alkaloids, and sugar amalgams as phytoconstituents. The leaf extract of Pepper (*Piper nigrum*) was also used as reducing as well as capping agent during the synthesis of AgNPs (Mallikarjuna, Sushma, Narasimha, Manoj, & Raju, 2014). On the other hand, the bark extract of *Saraca asoca* was used to synthesis AgNPs indicating the presence of hydroxyl, carboxyl, and amine functional groups containing phytoconstituents (Banerjee & Nath, 2015). The fruit extracts of *Malus domestica* was also used as a reducing agent for the synthesis of AgNPs (Ahmad et al., 2019). Furthermore, the peels of *Solanum nigrum*, *Nitraria schoberi*, *Vitis vinifera*, *Adansonia digitate*, and *Andean blackberry* have also been utilized for the synthesis of AgNPs (Ahmad et al., 2019). Besides these, the seed, and root extracts of different plants, natural rubber, polysaccharide, soluble starch, cinnamon, red apple, egg white, lemon grass, coffee, black tea, and *Abelmoschus esculentus* juice was reported as reducing agents for the synthesis AgNPs (Ahmad et al., 2019).

Microorganisms including fungi, bacteria, and yeast have also been used for the synthesis of AgNPs (Zhao et al., 2018). For example, AgNPs were synthesized both intracellularly and extracellularly using *Actinobacteria Rhodococcus* sp. NCIM 2891, and *Bacillus subtillus* IA751, respectively (Kannan & Subbalaxmi, 2011; Zhao et al., 2018). Several microalgae species including *Chaetoceros calcitrans*, *C. salina*, *Isochrysis galbana*, and *Tetraselmis gracilis* were used for the synthesis of AgNPs (Bahmani, Zargaran, Rafieian-Kopaei, & Saki, 2014). The marine algae, *Cystophora moniliformis*, was used as a reducing as well as stabilizing agent for the synthesis of AgNPs (Prasad et al., 2013). However, the microorganisms mediated AgNPs synthesis has several disadvantages including culture contamination, lengthy procedure, and less control over the size of nanoparticles (Siddiqi, Husen, & Rao, 2018). Fungus-mediated synthesis of AgNPs were chosen over the bacterial species due to their better tolerance and metal-bioaccumulation property. *Fusarium oxysporum* was used for the synthesis of spherical shaped AgNPs having diameter 5–50 nm (Ahmed, Hamzah, & Maaroof, 2018) and *Aspergillus fumigatus* was used for the synthesis of spherical as well as triangular shaped AgNPs (Ahmed et al., 2018).

The actual target of biological synthesis of AgNPs is to deliberately decrease or eliminate chemical hazards for protecting the environment from pollutants by using Green chemistry to develop the methodology to design, manufacture and use of AgNPs. The following figure (Fig. 4.1) shows the applications of silver nanoparticles.

Overall, the advantages of the biological syntheses of AgNPs include:

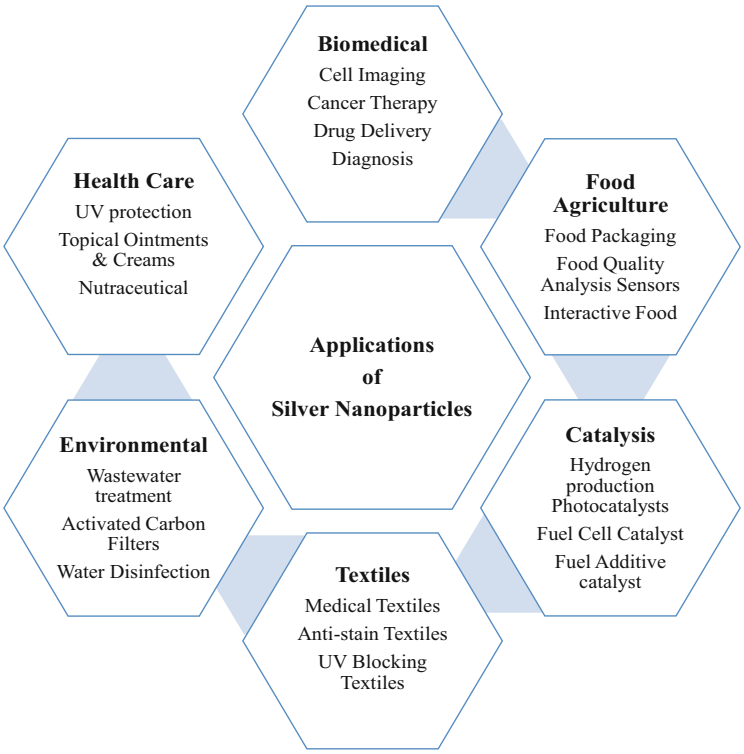

Fig. 4.1 The potential applications of silver nanoparticles

- Safe products
- Less waste
- Less accidents
- Cost effective
- Healthy working environment
- Compatible for pharmaceutical applications
- Large-scale production and
- Substantial energy saving processes.

4.2.2 Synthesis of Silver Nanoparticles Using Bacteria

One of the most cost effective approaches to control the composition as well as the shape of synthesized AgNPs is the use of bacterial strains in a periodic manner. Since bacteria have the propensity to reduce heavy metal ions, they could be used as a potential candidate for the synthesis of AgNPs. Some bacterial species can exploit their intrinsic defense mechanism to repress the toxic stresses of heavy metals or their ions (Bridges, Kidson, Lowbury, & Wilkins, 1979; Haefeli, Franklin, & Hardy,

1984). For example, *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, and *Pseudomonas aeruginosa* are able to remove metallic cations (i.e., Ag^+) from the culture medium as bacterial cells are capable of binding them strongly (Mullen et al., 1989).

A novel approach of synthesizing AgNPs using biological resources was studied by Saifuddin et al. by combining the supernatant of *Bacillus subtilis* culture and microwave irradiation in water (Saifuddin, Wong, & Yasumira, 2009). These NPs are stable in aqueous solution for up to 5–8 months at room temperature and in the dark. Several parameters including temperature, pH, light intensity, bacterial species, and concentration of salt (i.e., NaCl) influence the synthesis of AgNPs. For example, the supernatant of *A. kerguelensis* and *P. antarctica* culture don't produce AgNPs at the same temperature (Iravani, 2014). However, AgNPs showed significant antibacterial propensity against several multidrug resistant (MDR) bacterial strains including *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Klebsiella pneumoniae* (Priyadarshini, Gopinath, Priyadharsshini, Mubarakali, & Velusamy, 2013).

To synthesize AgNPs using bacteria, the bacteria were grown overnight in liquid medium at 37 °C and 120 rpm until the optical density (OD_{600}) of the culture reached ~0.6. The bacterial culture was then filtered to collect the supernatant that was used as a reducing agent to reduce $AgNO_3$ solution (1–10 mM). The mixture of bacterial supernatant and $AgNO_3$ solution was incubated at 37 °C at 150 rpm in the dark for 48–60 h to allow the formation of the AgNPs (Baltazar-Encarnación, Escárcega-González, Vasto-Anzaldo, Cantú-Cárdenas, & Morones-Ramírez, 2019). Electron microscopy analysis confirmed the formation of AgNPs either on the surface of the cell membrane or inside the cytoplasm or outside the cells. The exact location of the synthesis of AgNPs depends on the location of the enzymes (i.e., either on the cytoplasmic membrane or outside the cytoplasm) for the bioreduction of metal ions (Korbekandi, Iravani, & Abbasi, 2012) (Table 4.1).

Javaid et al. also reported that the bacteria mediated synthesis of AgNPs can occur either inside the cell (intracellular) or outside the cell (extracellular) or both depending on the location of reduction of silver ions (Javaid, Oloketuyi, Khan, & Khan, 2018). In most of the cases, bacteria reduce Ag^+ ions into their elemental form (i.e., Ag^0) that accumulated outside the cell. Bacterial cell wall proteins and secreted soluble enzymes act as reducing agents in the chemical conversion. Depending upon the composition of bacterial culture media, various shaped AgNPs were formed including spherical, hexagonal, triangular, disc, and cuboidal (Elbeshehy, Elazzazy, & Aggelis, 2015; Nanda & Saravanan, 2009; Otari et al., 2015; Oves et al., 2013). Extracellular synthesis facilitates easy recovery of AgNPs through high-speed centrifugation (~10,000 rpm) where AgNPs present in solution are trapped into cell pellet which can be resuspended using any preferable solvent (Javaid et al., 2018). For example, gram positive *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus persicus* produce AgNPs in the extracellular environment (Elbeshehy et al., 2015). However, for the intracellular synthesis of AgNPs, silver ions transport inside the bacterial cell through the membrane associated proteins. According to a report in 2014, Ag^0 are usually gathered together on the bacterial cell wall is approximately 25% of their mass (Murugan, Senthilkumar, Senbagam, & Al-Sohaibani, 2014).

Table 4.1 Biogenic synthesis of AgNPs using bacteria

Bacteria	Size (nm)	Shape	References
Acinetobacter calcoaceticus	8–12	Spherical	Singh et al. (2013)
Aeromonas sp.	6.4	Face-centered cubic	Singh, Du, and Yi (2017)
Acetobacter xylinum NCIM2526	–	Fiber	Ahmed, Kalla, Uppuluri, and Anbazhagan (2014)
Bordetella sp.	63–90	–	Thomas, Jasim, Mathew, and Radhakrishnan (2012)
Brevibacterium frigoritolerans strain DC2	97	Spherical	Singh et al. (2015)
Brevibacterium casei	10–50	Spherical	Kalishwaralal et al. (2010)
Bacillus sp.	5–15	–	Pugazhenthiran et al. (2009)
B. thuringiensis	43.52–142.97	–	Banu, Balasubramanian, and Moorthi (2014)
B. subtilis	20–60	Triangular, hexagonal	Kannan, Mukunthan, and Balaji (2011)
B. subtilis	60	Spherical	Sathiyanarayanan, Kiran, and Selvin (2013)
B. subtilis	5–60	Spherical	Saifuddin et al. (2009)
B. subtilis ANR 88	4–18	Spherical	Rane, Baikar, Ravi Kumar, and Deopurkar (2017)
B. magaterium	15–50	Hexagonal and cubical	Zaki, El Kady, and Abd-El-Haleem (2011)
B. thuringiensis	20–40	–	Verma et al. (2018)
Deinococcus radiodurans	4–50	Spherical	Kulkarni, Shaiwale, Deobagkar, and Deobagkar (2015)
Escherichia coli	20–50	Spherical	Kushwaha, Singh, Bhartariya, Singh, and Yasmeen (2015), Verma et al. (2018)
	42.2–89.6	Spherical	Chumpol and Siri (2018)
Enterobacter cloacae	28.5–122	Spherical	Shahverdi, Fakhimi, Shahverdi, and Minaian (2007)
Enterobacter aerogenes	25–35	Spherical	Karthik and Radha (2012)
Gluconobacter roseus	10	–	Krishnaraj and Berchmans (2013)
Idiomarina sp.	25	–	Seshadri, Prakash, and Kowshik (2012)
Klebsiella pneumonia	15–37	Spherical	Kalpana and Lee (2013)
	5–32		
K. oxytoca DSM 29614	–	Spherical	Baldi et al. (2016)

(continued)

Table 4.1 (continued)

Bacteria	Size (nm)	Shape	References
Lactobacillus sp.	50	–	Brayner et al. (2007)
L. mindensis	2–20	Spherical	Dhoondia and Chakraborty (2012)
Microbacterium resistens	10–20	Spherical	Wang et al. (2016)
Moganella sp.	10–40	Quasi-spherical	Parikh et al. (2011)
Nocardiopsis valliformis strain OT1	5–50	Spherical	Rathod, Golinska, Wypij, Dahm, and Rai (2016)
Novosphingobium sp. THG-C3	8–25	Spherical	Du, Singh, and Yi (2017)
Ochrobactrum anthropi PH5	35–85	Spherical	Gahlawat et al. (2016), Thomas et al. (2014)
Proteus mirabilis	10–20	Spherical	Samadi et al. (2009)
Pseudomonas deceptionensis DC5	127	Spherical	Jo et al. (2016)
P. aeruginosa	6.3 ± 4.9	Spherical, disc shaped	Srivastava and Constanti (2012)
	8–24	Spherical	
	5–25	Quasispherical	
P. fluorescens JP2 *P. stutzeri* AG259	5–60	Spherical	Klaus, Joerger, Olsson, and Granqvist (1999)
P. aeruginosa SN5	35–60	–	Naik, Prabhu, Samant, Naik, and Shirodkar (2017)
P. aeruginosa DM1	45–100	Spherical Face-centered cubic	Kumari, Barsainya, and Singh (2017)
Pseudomonas sp.	50	Variable	Punjabi, Yedurkar, Doshi, Deshapnde, and Vaidya (2017)
Pedicoccus pentosaceus	–	–	Sintubin et al. (2009)
Rhodococcus sp.	10–15	Spherical	Otari, Patil, Ghosh, Thorat, and Pawar (2015)
Rhodopseudomonas palustris	5–20	Spherical	Chai and Bai (2010)
Rhodobacter spheroids	3–15	Spherical	Bai et al. (2011)
Streptomyces sp. 09 PBT 005	198–595	Spherical	Kumar et al. (2015)
Streptomyces sp. SS2	67.95 ± 18.52	Spherical	Mohanta and Behera (2014)
Streptomyces glaucus	4–25	Spherical	Tsibakhashvili et al. (2011)
Streptomyces hygroscopicus	20–30	Spherical	Sadhasivam, Shanmugam, and Yun (2010)

(continued)

Table 4.1 (continued)

Bacteria	Size (nm)	Shape	References
Sinomonas mesophila MPKL 26	4–50	Spherical	Manikprabhu et al. (2016)
Sporosarcina koreensis DC4	–	Spherical	Singh et al. (2016)
Streptacidiphilus durhamensis HGG16n	8–48	Spherical	Buszewski et al. (2018)
Streptacidiphilus sp. CGG11n	4–45	Spherical	Railean-Plugaru et al. (2016)
Staphylococcus aureus	160–180	Spherical	Nanda and Saravanan (2009)
Staphylococcus aureus Staphylococcus epidermidis	<60	Spherical, oval, rod, and triangular	Amin, Khashyarmanesh, and Bazzaz (2016)
Streptococcus thermophiles	28–122	Spherical	El-Shanshoury, Elsilk, and Ebeid (2011)
Stenotrophomonas maltophilia	93	Cuboidal	Oves et al. (2013)
Shewanella oneidensis MR-1	2–16 20 ± 3	Spherical	Debabov et al. (2013), Ramasamy, Lee, and Lee (2016)
Serratia nematodiphila	10–31	Spherical	Shahverdi et al. (2007)
Vibrio alginolyticus	50–100	Spherical	Rajeshkumar and Malarkodi (2014)
Weissella oryzae DC6	10–30	Spherical	Singh, Kim, Wang, Mathiyalagan, and Yang (2016)
Xanthomonas oryzae	14.86	Spherical, triangular, and rod shaped	Narayanan and Sakthivel (2013)

Although silver has fatal impact on bacteria, the silver resistant strains can overcome it by reducing Ag^{+} to Ag^{0}. For example, *Corynebacterium* sp. SH09 produces AgNPs (10–15 nm), which forms diamine Ag complex on cell wall (Zhang et al., 2005). The recovery of intracellularly synthesized AgNPs requires few additional steps including the lysis of bacteria using ultra-sonication (Kalishwaralal, Barathmanikanth, Pandian, Deepak, & Gurunathan, 2010), heat treatment, or by chemicals (Fesharaki et al., 2010).

4.2.3 Synthesis of Silver Nanoparticles Using Fungi

Since fungi have higher intrinsic metal tolerance and bioaccumulation limit, they play a major role in the synthesis of metallic nanoparticles, especially AgNPs. The fungus-mediated synthesis of AgNPs has several advantages including very high intracellular

metal uptake capacity, easy scale-up method, financial feasibility, large biomass, large amount of enzymes are produced per unit of biomass, simple downstream approach, easy recovery of biomass, and fungi secreted reductase facilitates the formation of metallic nanoparticle (Sastry, Ahmad, Khan, & Kumar, 2003). Furthermore, fungi secrete huge amount of proteins which are responsible for the high yield of nanoparticles (Mohanpuria, Rana, & Yadav, 2008; Volesky & Holan, 1995).

The synthesis process of $AgNO_3$ involves two major steps: (1) bioreduction of $AgNO_3$ to produce AgNPs; and (2) simultaneous stabilization of the particles for a certain period of time by a suitable capping agent. The fungal strains *Penicillium*, and *Aspergillus* quickly produced extracellular AgNPs (Bhainsa & D'souza, 2006). The isolated fungal strain was cultured in a liquid medium at optimal conditions. After 60–72 h of incubation, the biomass was filtered and washed several times with sterile water to remove any contaminants (e.g., sucrose) during the synthesis of nanoparticles. *Penicillium* is a common biomass and has the property to enhance the cost-effective preparation of AgNPs. Fungal biomass need to be accumulated within 72 h to produce AgNPs. Li et al. established a method to prepare AgNPs at room temperature by reduction of Ag^+ with the culture supernatants of *Aspergillus terreus* (Li et al., 2012). Here, NADH performed the act of a reducing agent. *Fusarium acuminatum* and *F. solani* have also been studied extensively for the formation of AgNPs (Ingle, Gade, Pierrat, Sonnichsen, & Rai, 2008) (Table 4.2).

At a glance, the synthesis of AgNPs was confirmed by the change of color of fungal culture supernatant from light yellow to brown within few hours of incubation due to the surface plasmon resonance (SPR) effect of the nanoparticles and the reduction of $AgNO_3$ (Sastry et al., 2003; Wiley et al., 2006). Proteins excreted by the fungus keep these particles stable for few months in aqueous solution (Ahmad et al., 2003). Functional groups (e.g., ≡C–O–C≡, =C=O, ≡C–O–R and =C=C=) analysed through FTIR suggest that amino groups present in the fungal proteins act as a capping agent during the reaction process (Ingle et al., 2009). Microscopic analysis of the fungal cells confirmed that the synthesized AgNPs were formed beneath the cell wall layer because of the presence of membrane proteins that act as capping agent in the reduction reaction. Moreover, AgNPs are stable at room temperature for up to 4 months.

4.2.4 Synthesis of Silver Nanoparticles Using Algae

Stable colloidal AgNPs have been synthesized using algae as a source of reducing agent. Recent studies have found that AgNPs were synthesized at room temperature using *Spirulina platensis* and *Nostoc* sp. (Abdelghany et al., 2018). Various strains of microalgae including *Botryococcus braunii*, *Coelastrum* sp., *Spirulina* sp., and *Limnothrix* sp. were used to synthesize AgNPs with a diameter of 15.67, 19.28, 13.85 and 25.65 nm, respectively (Patel, Berthold, Puranik, & Gantar, 2015). Annamalai et al. described the biosynthesis of AgNPs using the aqueous extract of *Chlorella vulgaris* as reducing agent (Annamalai & Nallamuthu, 2016). The formation of AgNPs within few minutes of mixing silver nitrate solution with the extract of

Table 4.2 Biogenic synthesis of AgNPs using fungi

Name of fungi	Size range (nm)	Reference
Aspergillus flavus Johann Heinrich Friedrich Link	8.92 nm	Vigneshwaran, Kathe, Varadarajan, Nachane, and Balasubramanya (2007)
Aspergillus fumigatus	5–25	Bhainsa and D'souza (2006)
Aspergillus fumigatus Fresenius	5–25 nm, monodispersed	Bhainsa and D'souza (2006), Prabhu et al. (2009)
Aspergillus niger van Tieghem	20 nm, spherical	Gade et al. (2008)
Alternaria alternate (Fr.) Keissl	20–60 nm polydisperse spherical	Gajbhiye, Kesharwani, Ingle, Gade, and Rai (2009)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	10–100 nm, spherical	Balaji et al. (2009)
Coriolus versicolor (L.) Quél. Silver	25–75 nm, spherical	Sanghi and Verma (2009)
Fusarium oxysporum Schlecht. em. Snyder and Hansen	5–50 nm	Senapati et al. (2004)
Fusarium oxysporum Schlecht. em. Snyder and Hansen	8–14 nm	Senapati, Ahmad, Khan, Sastry, and Kumar (2005)
Fusarium solani (USM-3799) (Mart.) Sacc.	16.23 nm, spherical	Ingle, Rai, Gade, and Bawaskar (2009)
Fusarium semitectum Berk. and Ravenel	10–60 nm, spherical	Basavaraja, Balaji, Lagashetty, Rajasab, and Venkataraman (2008)
Phoma sp. 3.2883	71.06–74.46	Chen, Lin, and Ma (2003)
Phaenerochaete chrysosporium	50–200	Vigneshwaran, Kathe, Varadarajan, Nachane, and Balasubramanya (2006)
Phanerochaete chrysosporium Burdsall	5–200 nm, pyramidal	Vigneshwaran et al. (2006)
Penicillium brevicompactum WA2315 Dierckx	58.35 ± 17.88 nm	Shaligram et al. (2009)
Penicillium fellutanum Biourge	5–25 nm, spherical	Kathiresan, Manivannan, Nabeel, and Dhivya (2009)
Phoma glomerata (Corda) Wollen w. & Hochapfel.	60–80 nm, spherical	Birla et al. (2009)
Rhizopus nigricans Ehrenberg	35–48 nm, polydispersed and spherical	Ravindra and Rajasab (2014)
Trichoderma viride Pers.	5–40 nm	Fayaz et al. (2010)
Trichoderma asperellum	13–18	Mukherjee et al. (2008)
Volvariella volvacea (Bulliard ex Fri) Singer	15 nm, spherical	Philip (2009)
Verticillium sp.	25 ± 12	Mukherjee et al. (2001)

Pithophora oedogonium algae was described by Sinha *et al.* (Sinha, Paul, Halder, Sengupta, & Patra, 2015). Microalgae including *Chaetoceros calcitrans*, *C. salina*, *Isochrysis galbana*, and *Tetraselmis gracilis* were also used to synthesize AgNPs (Rajput, 2015). Prasad et al. used marine algae such as *Cystophora moniliformis* as a reducing as well as stabilizing agent to synthesize AgNPs (Prasad et al., 2013). Table 4.3 summarizes the application of micro algae, macro algae, green algae, brown algae, red algae, and blue-green algae in the synthesis of AgNPs.

4.2.5 Synthesis of Silver Nanoparticles Using Plants

The plant extracts have been used in the green synthesis of nanoparticles. It offers better control over the crystal growth, stability, biocompatibility, toxicity, and cost effectiveness (Mohanpuria et al., 2008; Saifuddin et al., 2009; Verma, Kharwar, & Gange, 2010; Willner, Basnar, & Willner, 2007). Plant extracts are attractive platform to synthesize nanocrystals because they are nontoxic and act as capping as well as stabilizing agents. It is easy to grow plants that also reduce the cost of culture media required in microbial approaches (Sharma et al., 2009; Singhal, Bhavesh, Kasariya, Sharma, & Singh, 2011). In this approach, plant extracts have been used both as capping and reducing agents for the synthesis of nanoparticles (Prasad, 2014). The concentration of plant extracts play a vital role to maintain the size and shape of the nanoparticles. The water soluble heterocyclic, and polyol compounds present in the plant extracts are the major acting elements for the reduction and stabilization of silver ions in AgNPs. During the process, $AgNO_3$ was reduced to synthesize the silver particles due to the presence of various proteins (Huang et al., 2007).

Table 4.4 summarizes the plants belonging to various families and have the potential to synthesize AgNPs of diverse shape, and size and their applications.

Different concentrations of $AgNO_3$ solution was mixed with different concentrations of plant extracts to prepare AgNPs of different size and shape (Ahmed, Saifullah, Swami, & Ikram, 2016). The reaction mixture was kept in amber condition to avoid the photo-activation of silver nitrate at room temperature. The change of the color of solution from colorless to brown confirms the reduction of Ag^+ to Ag^0.

Although water is the most preferred solvent for the extraction of phytoconstituents, several organic solvents (e.g., methanol, ethanol, hexane, and ethylacetate) are also used in the extraction process (Kulkarni, Srivastava, Nagalgaon, & Zunjarrao, 2012; Logeswari, Silambarasan, & Abraham, 2015; Patete et al., 2011; Rahimi-Nasrabadi, Pourmortazavi, Shandiz, Ahmadi, & Batooli, 2014; Rajesh, Raja, Rathi, & Sahayaraj, 2012; Sadeghi & Gholamhoseinpoor, 2015; Sadeghi, Rostami, & Momeni, 2015; Shafaghat, 2015). Different parts of plants including leaves, fruits, seeds, and stems containing antioxidants, and carbohydrates are used in the generation of AgNPs to avoid hazardous chemicals (e.g., sodium borohydride) used in chemical synthesis process (Hebbalalu, Lalley, Nadagouda, & Varma, 2013). The critical parameters involved in the whole process include pH of

Table 4.3 Algae-mediated synthesis of AgNPs

Name of algae	Size (nm) of AgNPs	Shape	Type of algae	References
Gelidium amansii	27–54	Spherical	Macroalgae	Pugazhendhi, Prabakar, Jacob, Karuppusamy, and Saratale (2018)
Chaetomorpha linum	3–44	Varied	Macroalgae	Kannan, Arumugam, Ramya, Manivannan, and Anantharaman (2013)
Colpmenia sinusa	20	Spherical	Macroalgae	El-Rafie et al. (2013)
Ulva fasciata	12	Spherical	Macroalgae	El-Rafie et al. (2013)
Jania rubins	7	Spherical	Macroalgae	El-Rafie et al. (2013)
Pterocladia capillacae	7	Spherical	Macroalgae	El-Rafie et al. (2013)
Caulerpa racemose	10	Spherical	Macroalgae	McDonald et al. (2011)
Sargassum wightii	8–27	Spherical	Macroalgae	Govindaraju, Kiruthiga, Kumar, and Singaravelu (2009)
Chaetoceros calcitrant	53.1–73.9	NA	Microalgae	Merin, Prakash, and Bhimba (2010)
Chlorella salina	53.1–73.9	NA	Microalgae	Merin et al. (2010)
Isochrysis galbana	53.1–73.9	NA	Microalgae	Merin et al. (2010)
Tetraselmis gracilis	53.1–73.9	NA	Microalgae	Merin et al. (2010)
Ulva compressa	66.3	Cubic	Green algae	Minhas et al. (2018)
Cladophora glomerata	81.8	Cubic	Green algae	Minhas et al. (2018)
Caulerpa serrulata	10	Spherical–ellipsoidal	Green algae	Aboelfetoh, El-Shenody, and Ghobara (2017)
Enteromorpha compressa	4–24	Spherical	Green algae	Ramkumar et al. (2017)
Spirogyra varians	35	Quasi-spheres	Green algae	Salari, Danafar, Dabaghi, and Ataei (2016)
Caulerpa racemose	10	Spherical	Green algae	Kathiraven, Sundaramanickam, Shanmugam, and Balasubramanian (2015)
Chlorella pyrenoidosa	2–15	Spherical	Green algae	Aziz et al. (2015)
Enteromorpha flexuosa	2–32	Spherical	Green algae	Yousefzadi, Rahimi, and Ghafori (2014)
Spatoglossum asperum	22–60	Spherical	Brown algae	Ravichandran et al. (2018)
Saccharina japonica	14.77	Spherical	Brown algae	Sivagnanam et al. (2017)

(continued)

Table 4.3 (continued)

Name of algae	Size (nm) of AgNPs	Shape	Type of algae	References
Ecklonia cava	43	Spherical	Brown algae	Inbakandan, Venkatesan, and Khan (2010)
Sargassum longifolium	NA	Spherical and ellipsoidal	Brown algae	Kathiraven et al. (2015)
Colpomenia sinuosa	15–35	Spherical	Brown algae	Roy, Bulut, Some, Mandal, and Yilmaz (2019)
Gelidium amansii	27–54	Spherical	Red algae	Pugazhendhi et al. (2018)
Spyridia fusiformis	5–50	Spherical	Red algae	Murugesan, Bhuvaneswari, and Sivamurugan (2017)
Gracilaria birdiae	20.2–94.9	Spherical	Red algae	de Aragao et al. (2019)
Gracilaria crassa	60–200	Spherical	Red algae	Lavakumar et al. (2015)
Gracilaria corticata	18–46	Spherical	Red algae	Kumar, Selvi, and Govindaraju (2013)
Pterocladia capillacea	7–18	Spherical	Red algae	Roy et al. (2019)
Jania rubens	5–20	Spherical	Red algae	Roy et al. (2019)
Porphyra vietnamensis	13	Spherical	Red algae	Venkatpurwar and Pokharkar (2011)
Nostoc sp.	51–100	Spherical	Blue-green algae	Sonker, Pathak, Kannaujiya, and Sinha (2017)
Synechococcus sp.	140	Spherical	Blue-green algae	Keskin, Oya, Koçberber Kılıç, Dönmez, and Tekinay (2016)
Anabaena sp.	24.13	Irregular	Blue-green algae	Patel et al. (2015)
Limnothrix sp.	31.86	Elongated	Blue-green algae	Patel et al. (2015)
Synechocystis sp.	14.64	Irregular	Blue-green algae	Patel et al. (2015)
Anabaena doliolum	10–50	Spherical	Blue-green algae	Singh et al. (2014)
Nostoc commune	15–54	Spherical	Blue-green algae	Morsy, Nafady, Abd-Alla, and Elhady (2014)

solution, temperature, extract composition, silver concentration, reaction time, and speed of agitation (Kora, Sashidhar, & Arunachalam, 2010). The carboxylate ions and AgNPs provide the stability in together (Philip, 2010). UV-vis spectroscopy confirms the formation of (Babu & Prabu, 2011). However, SEM, TEM, FTIR, and XRD analysis show the morphology, and crystalline structure of the nanoparticles.

Table 4.4 Biogenic synthesis of Ag NPs using plants

Plant species	Plant parts	Size (nm)	Reference
Allium cepa	Extract	–	Saxena, Tripathi, and Singh (2010)
Aloe vera	Leaf	15–15.6	Chandran, Chaudhary, Pasricha, Ahmad, and Sastry (2006)
Argemone mexicana	Leaf	–	Singh, Jain, Upadhyay, Khandelwal, and Verma (2010)
Azadirachta indica	Leaf	50–100	Shankar, Rai, Ahmad, and Sastry (2004)
Bacopa monniera	Leaf	15–120	Mahitha et al. (2011)
Boswelliaovali foliolata	Bark and leaf	–	Ankanna, Prasad, Elumalai, and Savithramma (2010)
Capsicum annuum	Leaf	15–20	Li et al. (2007)
Cinnamomum camphora	Leaf	55–80	Huang et al. (2007)
Cycas circinalis	Leaf	2–6	Jha and Prasad (2010)
Dalbergia sissoo	Leaf	5–55	Singh, Baboota, Naik, and Singh (2012)
Emblica officinalis	Leaf	10–20	Ankamwar, Damle, Ahmad, and Sastry (2005)
Euphorbia hirta	Leaf and bark	–	Elumalai et al. (2010)
Ficus benghalensis	Leaf	16	Saxena, Tripathi, Zafar, and Singh (2012)
Gliricidia sepium	Leaf	10–50	Raut Rajesh, Lakkakula Jaya, Kolekar Niranjan, Mendhulkar Vijay, and Kashid Sahebrao (2009)
Ipomea carnea	Leaf	30–130	Daniel et al. (2014)
Jatropha curcas	Latex	10–20	Bar et al. (2009)
Medicago sativa	Leaf	20–40	Gardea-Torresdey et al. (2003)
Memecylonedule	Leaf	50–90	Elavazhagan and Arunachalam (2011)
Murraya koenigii	Leaf	10–25	Christensen, Vivekanandhan, Misra, and Mohanty (2011)
Nerium indicum	Leaf	–	Priya, Selvi, and Paul (2011)
Nicotiana tobaccum	Leaf	8	Prasad et al. (2011)
Ocimum sanctum	Leaf	–	Singhal et al. (2011)
Pelargonium graveolens	Leaf	16–40	Shankar, Ahmad, and Sastry (2003)

(continued)

Table 4.4 (continued)

Plant species	Plant parts	Size (nm)	Reference
Phyllanthus maderaspatensis	Leaf	59–76	Annamalai, Christina, Christina, and Lakshmi (2014)
Rhizophora apiculata	Leaf	13–19	Antony et al. (2011)
Santalum album	Leaf	80–200	Swamy and Prasad (2012), Swamy, and Varma (2012)
Saraca indica	Leaf	13–50	Tripathi, Ranac, Shrivastav, Singh, and Shrivastav (2013)
Sorghum bicolor	Bran	–	Njagi et al. (2011)
Syzgium cumini	Leaf	100–160	Prasad et al. (2012)
Syzgium cumini	Bark	20–60	Prasad and Swamy (2013)
Trapa bispinosa	Peel	–	Pandey et al. (2013)
Vitex negundo	Leaf	–	Zargar et al. (2011)

4.3 Therapeutic Applications of Biogenic Silver Nanoparticles

4.3.1 Antibacterial Activity

Biogenic AgNPs (bAgNPs) showed excellent antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria including multidrug resistant bacteria. The antibacterial propensity of bAgNPs depends on their shape, size, and concentration. Several examples of the application of bAgNPs are in distillation techniques, surgical devices, coating of bone prostheses and dental composites (Percival, Bowler, & Dolman, 2007). In case of Gram-positive bacteria, the cell wall is mainly composed of peptidoglycan layer which is further crosslinked by oligopeptides that makes the penetration of bAgNPs difficult. However, bAgNPs can easily penetrate through the relatively thin peptidoglycan layer of the cell wall of Gram-negative bacteria and thus, destabilizes the outer membrane (Shrivastava et al., 2007).

Biogenic AgNPs demonstrate antibacterial activity through several mechanisms (showed in Fig. 4.2) including: (1) damaging bacterial cell membrane through formation of several depths and gaps (Roy et al., 2019); (2) increasing cell membrane permeability due to interactions of silver ions with the bacterial membrane through either noncovalent interactions or molecular crowding, (Ranjan Sarker et al., 2019); (3) generation of oxidative stress due to reactive oxygen species (ROS) (Hossain et al., 2019); and (4) disruption of energy metabolism and inhibition of gene transcription (Roy et al., 2019). Once silver ions from bAgNPs are released, they interact with the sulfur- and phosphorus-containing proteins present in the plasma membrane as well as in the bacterial cell wall through electrostatic

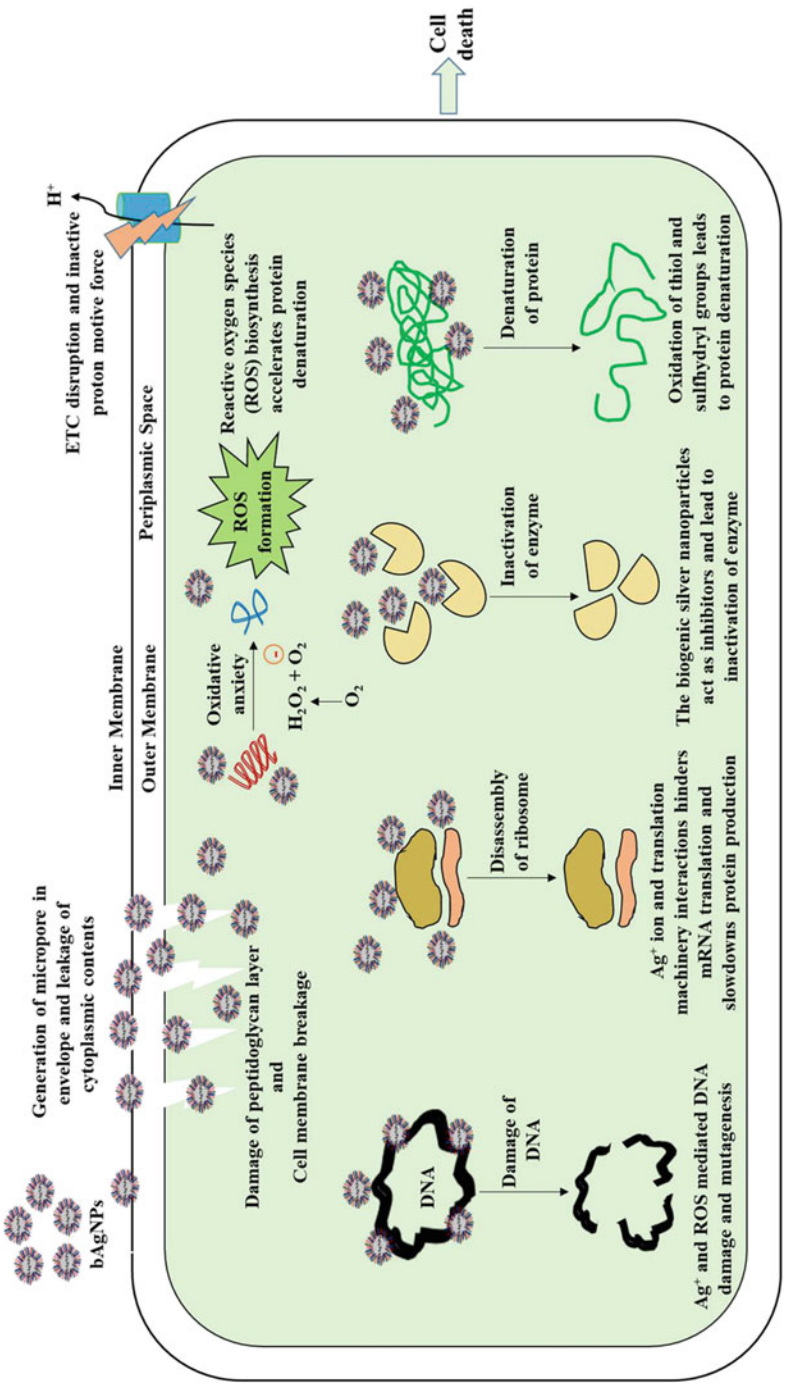

Fig. 4.2 Anti-bacterial mechanism of biogenic silver nanoparticles (bAgNPs)

interactions. This leads to the formation of pores in the cell membrane through which bacterial intracellular contents are discharged to the extracellular space that result in permanent cell wall damage as well as electrochemical imbalance between the cytosol and extracellular spaces (Hossain et al., 2019). Furthermore, bAgNPs also interfere with the signal transduction pathways of bacteria through dephosphorylating the tyrosine residue of certain peptides (i.e., tyrosine kinase and phosphatases) and prevent the growth of bacteria (Prabhu & Poulose, 2012). Biogenic AgNPs also bind to the plasmalemma and arrest the respiratory tract as well as the electron transport chain of bacteria before interfering with the ATP synthesis (Bragg & Rainnie, 1974). The bAgNPs show the strongest antibacterial activity because of their large surface area to volume ratios.

In this chapter, the antibacterial activity of plant-mediated bAgNPs against both the Gram-positive and Gram-negative bacteria has been discussed in detail. Recently, Hossain et al. (2019) described the synthesis of aqueous biogenic AgNPs (Aq-bAgNPs) and ethanolic biogenic AgNPs (Et-bAgNPs) using the aqueous and ethanolic extracts, respectively, of *Andrographis paniculata* stem. Both the bAgNPs showed excellent antibacterial activity against several Gram-positive as well as Gram-negative bacteria including enteropathogenic *Escherichia coli* (EPEC), *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio cholerae*, *Enterococcus faecalis*, *Hafnia alvei*, *Acinetobacter baumannii*, *E. coli DH5α*, *E. coli K12*, *and Bacillus subtilis.* The highest antibacterial activity of Aq-bAgNPs and Et-bAgNPs was reported against *S. aureus*, a Gram-positive bacteria, and the zones of inhibition (ZOI) were ~25 and 28 mm in diameter, respectively. Moreover, both the bAgNPs showed better antibacterial activity against Gram-positive bacteria (i.e., *S. aureus*) when compared to that Gram-negative bacteria (i.e., EPEC). This is because the cell wall of Gram-positive bacteria contains a thick peptidoglycan layer and plentiful pores through which external molecules can enter into the cells and bring about membrane damage and cellular death (Bu et al., 2010; Hossain et al., 2019).

4.3.2 Antifungal Activity

Fungi are considered as potent skin parasites and they are mainly responsible for skin as well as hair infections (Moazeni, Rashidi, Shahverdi, Noorbakhsh, & Rezaie, 2012). Immunocompromised patients are more likely to be infected by fungal species (Kim et al., 2008). It has been reported that bAgNPs demonstrate several medicinal applications including antifungal activity (Gajbhiye et al., 2009; Kumar et al., 2013; Mallmann et al., 2015). The green synthesized AgNPs are highly effective against infectious diseases due to their potent antifungal activity when compared to the azole drugs which are toxic at higher concentrations (Bankar, Joshi, Kumar, & Zinjarde, 2010; Gajbhiye et al., 2009; Kim et al., 2009; Kumar et al., 2013; Vivek, Kumar, Steffi, & Sudha, 2011). Biogenic silver nanoparticles having potent antifungal activity can be a safe and cost effective approach for the treatment of fungal infections and to control the fungal growth as well (Ashajyothi, Prabhurajeshwar, Handral, & Kelmani, 2016).

The biologically synthesized AgNPs can be used as effective agents against different fungal pathogens (Sawada et al., 2012). The phytoconstituents act as capping agents and bind to the surface of the AgNPs via noncovalent interactions. The capping agents prevent the aggregation of nanoparticles and make them stable (Prabhu & Poulose, 2012). These bAgNPs have enhanced antifungal activity because of their size. It has been reported that smaller size particles can penetrate easily and quickly through the cell membrane pores (Martínez-Castañon, Nino-Martinez, Martinez-Gutierrez, Martinez-Mendoza, & Ruiz, 2008). Hence, bAgNPs have elevated antifungal activity.

The antifungal activity depends on the attachment as well as the interaction of bAgNPs with the thiol groups of membrane proteins (Fig. 4.3) (Durán, Marcato, Ingle, Gade, & Rai, 2010). Biogenic AgNPs retard fungal growth by producing reactive oxygen species (ROS) (Saini, Saha, Roy, Chowdhury, & Babu, 2016) and disrupting fungal cell membrane (Vazquez-Muñoz, Avalos-Borja, & Castro-Longoria, 2014) that bring about the inhibition of various enzymes (Wigginton et al., 2010) and alteration of DNA replication (Bhabra et al., 2009). Biogenic AgNPs also interfere with the fungal membrane integrity, respiration, and the release of cellular contents to the extracellular environment. More specifically, bAgNPs bring about antifungal activity in two ways after their interaction with the fungi. Firstly, the nanoparticles release toxic Ag^+ ions into the cytosol and inhibit the activity of enzymes (i.e., protein kinase A and C, phospholipases, chitin enzymes etc.) (Wang, Hu, & Shao, 2017). Secondly, the membrane pores created by the nanoparticles alter the regular electron transport system and thus, inhibit the regular budding process (Wang et al., 2017).

Kumar et al. found that the enhanced antifungal activity of bAgNPs is due to the synergistic effect of silver ion in the treatment of systemic and surface fungal infections (Kumar & Poornachandra, 2015). This is because of the larger surface area of bAgNPs and the released ions from AgNPs bind to the sulfur containing membrane proteins. Inactivation of enzymes brings about oxidative stress for the cells and eventually alter the regular DNA replication process (Fatima, Verma, Pathak, & Bajpai, 2016; Rai et al., 2014; Reidy, Haase, Luch, Dawson, & Lynch, 2013). In addition, the concentration dependent activity of the Ag nanoparticles was also observed against *Aspergillus niger*, and *Trichoderma harzianum* (Guilger-Casagrande & De Lima, 2019).

4.3.3 Antioxidant Activity

Oxidative stress is the cause of many complex and fatal diseases including cancer, cardiac diseases as well as premature aging (Sreekanth, Ravikumar, & Eom, 2014). Cells undergo oxidative stress due to the formation of reactive oxygen species during the metabolic and respiratory processes. Plants contain important phytoconstituents including flavonoids, phenolics, tri-terpenoids, and coumarins compounds (Mittal, Kaler, & Banerjee, 2012). These secondary metabolites are responsible for the reduction of $AgNO_3$ to produce biogenic AgNPs and the secondary metabolites

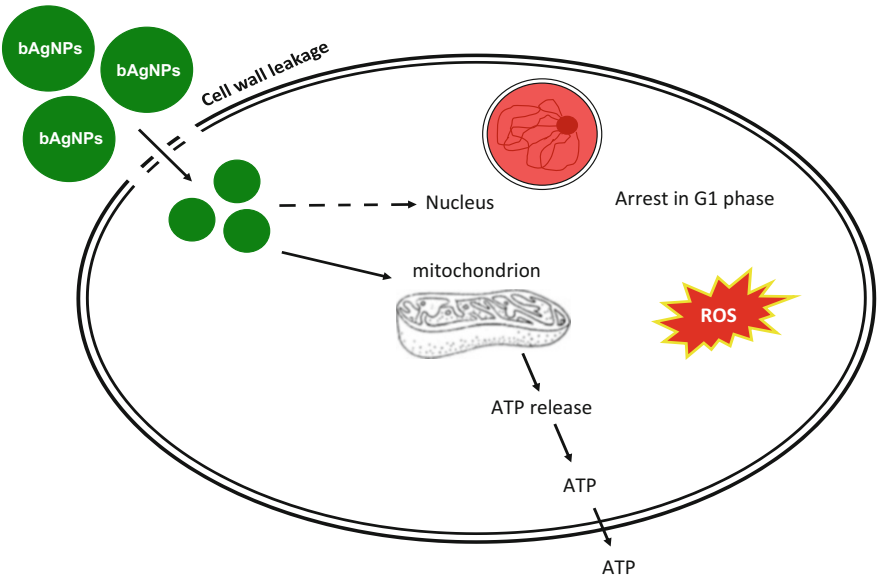

Fig. 4.3 Mechanism of antifungal activity of bAgNPs

act as capping agents to stabilize the particles. Biogenic AgNPs have antioxidant propensity due to the presence of phytoconstituents as capping agents (Mittal et al., 2012). The antioxidant potential of bAgNPs can be investigated through 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay (DPPH), Superoxide anion radical scavenging assay (SO), 2,20-Azin o-bis-(3-ethyl)benzothiazoline-6-sulfonic acid radical cation scavenging assay (ABTS), Reducing capacity assessment (RCA), and Ferric reducing antioxidant power assay (FRAP) (Moteriya & Chanda, 2018).

The phenolic compounds (e.g., ferulic acid, gallic acid, chlorogenic acid, catechin, and epicatechin) present in the plant extracts contribute directly to the antioxidant activity of bAgNPs (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003). This is because of the redox potential of phenolic compounds that act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Chang et al., 2001). Furthermore, possible mechanism of the antioxidant propensity of bAgNPs could be due to their electron donating ability (Morones et al., 2005). The antioxidant activity of bAgNPs could also be due to their small size, large surface area as well as ability to donate H^+ ions (Balan et al., 2016; Gurunathan et al., 2013; Thatoi et al., 2016).

4.3.4 Anticancer Activity

Among the other metal nanoparticles, silver nanoparticles play a key role in the delivery of drugs, anticancer therapy and imaging (Barabadi, Ovais, Shinwari, & Saravanan, 2017; Mukherjee, Chowdhury, Kotcherlakota, & Patra, 2014). Previous studies suggest that bAgNPs can be useful for anticancer therapy. Anticancer activity of bAgNPs was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Mosmann, 1983). The MTT assay is a colorimetric method for measuring cellular metabolic activity.

The bAgNPs showed anticancer activity against human breast cancer cells, MCF-7. For example, potential anticancer properties of AgNPs synthesized using *Chaenomeles sinensis* extracts against MCF-7 cells have been reported (Oh et al., 2018). Biogenic AgNPs synthesized using *Cibotium barometz* and *Chaenomeles sinensis* extracts also showed anticancer activity against MCF-7 cells (Oh et al., 2018; Wang et al., 2016). Abd Kelkawi et al. demonstrated the dose- and cell line-dependent anticancer activity of bAgNPs on HeLa and MCF-7 cancer cell lines using *Mentha pulegium* extract (Kelkawi, Kajani, & Bordbar, 2016). Biogenic AgNPs synthesized using rhizome extract of *Acorus calamus* demonstrated cytotoxicity against carcinoma cell line, A431 (Nayak, Pradhan, Ashe, Rauta, & Nayak, 2015).

It has been reported that the anticancer activity of bAgNPs depends mainly on the size, morphology and phytoconstituents (e.g., proteins, phenolic compounds, and flavonoids) coated around AgNPs. The thickness, surface hydrophobicity and surface charge distribution of nanoparticles also play important role on the anticancer potential of nanoparticles (Fröhlich, 2012). For example, some special morphological features were observed including the degradation of membrane integrity, decreased cell growth, cytoplasmic condensation and cell clumping in bAgNPs treated KB cells, whereas no modification was observed in untreated cells (Bhakya et al., 2016; Muthukrishnan, Kumar, & Rao, 2017).

Several studies confirm the anticancer activity of biogenic AgNPs (Pathania, Millard, & Neamati, 2009), although their exact mechanism of anticancer activity is yet to be elucidated. The bAgNPs demonstrate anticancer activity by disrupting the mitochondrial electron transport chain responsible for the production of reactive oxygen species and interfere with the synthesis of ATP (Pathania et al., 2009). The primary mechanism of the anticancer activity of AgNPs could be the release of Ag^+ from the colloidal AgNPs (Agnihotri, Mukherji, & Mukherji, 2013). It happens when the nanoparticles are spontaneously aggregated in the solution and internalized by cancer cells (Agnihotri et al., 2013). Ag^+ ions released from AgNPs can lead to cellular toxicity through various strategies including reduced cell membrane integrity and increased permeability, and increased cell apoptosis due to damaged DNA (Behboodi, Baghbani-Arani, Abdalan, & Shandiz, 2019). Biogenic AgNPs bring about the apoptosis of cancer cells through caspase-dependent and mitochondria-dependent pathways. The cellular uptake of bAgNPs could be through endocytosis, pinocytosis, and phagocytosis (Fig. 4.4). Once inside the cells, AgNPs trigger the production of reactive oxygen species (ROS), which bring about the up-regulation of

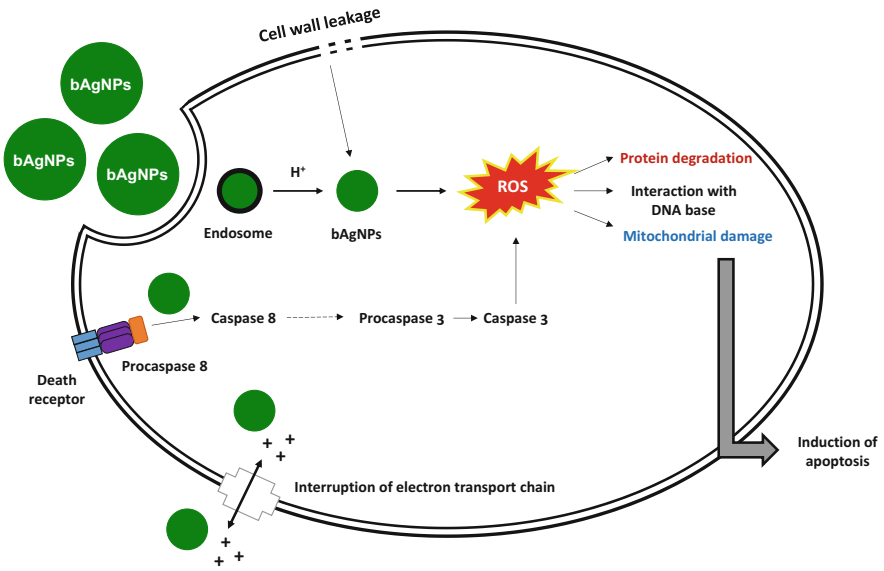

Fig. 4.4 Mechanism of anticancer activity of bAgNPs

the sequences of caspase-3 and p53 proteins. Caspase-3 plays a major role in cellular apoptosis by arresting G2/M phase of cell cycle (Barabadi et al., 2017; Ovais et al., 2016). The plausible explanations of the anticancer activity of bAgNPs were clarified through different assay methods using sensitive SKOV3 cells. Biogenic AgNPs cause oxidative stress to SKOV3 cells by producing reactive oxygen species (ROS), increasing the rate of lipid peroxidation (LPO), and decreasing the level of glutathione (GSH). Therefore, bAgNPs stimulate the apoptosis of SKOV3 cells by up-regulating caspase-3, caspase-8, caspase-9, p53, and BAX proteins, and down-regulating Bcl-2 antiapoptotic enzyme (Bethu, Netala, Domdi, Tartte, & Janapala, 2018). Abundance of ROS can influence the various processes in cells including change in gene expression, DNA damage, activation of transcription factors, and stimulation of apoptosis signal transduction pathway (Huang, Aronstam, Chen, & Huang, 2010). Biocompatibility of nanoparticles to healthy cells is also a vital step towards their clinical applications. Biogenic AgNPs have been considered nontoxic to normal CHO (Chinese hamster ovary) cells (Netala et al., 2018). They showed no cytotoxicity against CHO cells even at higher doses.

4.3.5 Antidiabetic Activity

Diabetes mellitus, a group of metabolic diseases and is characterized by the elevated blood sugar level over a long period of time, results from either the decreased insulin secretion or decreased insulin action or both. The main cause of Type-I diabetes mellitus is the deficiency of insulin secretion due to the destruction of insulin

producing pancreatic β-cells by β-cell specific autoimmune diseases. On the other hand, Type-II diabetes is caused by either the reduced insulin secretion or resistant to insulin action (Tabish, 2007). A therapeutic approach could be to reduce the elevated blood glucose level through inhibiting the carbohydrate digesting enzymes such as α-glucosidase and α-amylase. The inhibition of the enzymatic activity of α-glucosidase, and α-amylase prevents the breakdown of carbohydrates into monosaccharides, the main cause of hyperglycemia, that gets assimilated very easily into the body (Saratale et al., 2018). Hence, the synthetic compounds inhibiting the carbohydrate hydrolyzing enzymes help to control the hyperglycemia or elevated blood glucose level (Saratale et al., 2018). For example, Acarbose, a synthetic oligosaccharide, helps to prolong the digestion of carbohydrates by inhibiting the activity of pancreatic α-amylase (Swarnalatha, Rachela, Ranjan, & Baradwaj, 2012). Acarbose has many side effects including diarrhea, abdominal pain and soft feces (Swarnalatha et al., 2012). As a result, plant-based drugs are the most promising for the treatment of diabetes mellitus. Some herbs inhibit the activity of α-amylase to control the breakdown of carbohydrates as well as their absorption (Narkhede, Ajimire, Wagh, Mohan, & Shivashanmugam, 2011).

The peel extract of *Ananas comosus* (L.) was used to synthesize bAgNPs that showed promising antidiabetic propensity in a dose-dependent manner. The activity of α-glucosidase was inhibited almost 100% at a concentration as low as 1 μg/mL of bAgNPs. The inhibition of α-glucosidase activity is useful for the treatment of insulin independent diabetes due to slowing down of the release of glucose in the blood (Das, Patra, Debnath, Ansari, & Shin, 2019). The bAgNPs synthesized using herbal extract (*Psoralea corylifolia*) possess potent inhibitory activity against protein tyrosine phosphatases (PTPs) enzyme to slow down the breakdown of carbohydrates (Shanker, Mohan, Hussain, Jayarambabu, & Pravallika, 2017). The biogenic AgNPs synthesized using the extract of *Azadirachta indica* (neem) and *Aloe vera* showed potent antidiabetic propensity through inhibiting the pancreatic α-amylase activity which is better than that of acarbose (Sathvika, Rajeshkumar, Lakshmi, & Roy, 2019). Since bAgNPs are easy to synthesize, cost effective, and eco-friendly, they have a great potential to play an important role as the alternative to conventional antidiabetic drugs.

4.3.6 Wound Healing Activity

The antimicrobial and anti-inflammatory activity of AgNPs make them a unique therapeutic agent for wound healing in diabetic patients (Dai et al., 2016; Tian et al., 2007). Silver nanoparticles is considered as a potential topical therapeutic agent in wound care systems because of their antibacterial and anti-inflammatory potential that accelerate the wound healing process by preventing microbial contamination as well as modulating the expressions of the matrix metalloproteinases leading to complete epithelization in chronic wounds (Jain et al., 2009). Animals treated with AgNPs showed better wound-healing activity when compared to that of either negative- or positive-control groups. Al-Shmgani et al. reported the wound healing

potential of bAgNPs in streptozotocin-induced diabetic mice (Al-Shmgani, Mohammed, Sulaiman, & Saadoon, 2017). Biogenic AgNPs also showed positive response in terms of their antimicrobial potential, reduced inflammation through decreased infiltration of lymphocytes and mast cells, and modification of fibrogenic cytokines (Tian et al., 2007).

During the treatment process, AgNPs treated wound showed no evidence of microbial contamination, bleeding or pus formation, while wound without any treatment showed significant inflammatory response. The healing of bAgNPs treated skin-excision wound was rapid and it was almost covered by an epithelial layer. Bioactive materials such as silver-based compounds, which can change the cytokine cascade, may improve the appearance of wounds by immunomodulation (Edwards-Jones, 2012). The reduced wound size and increased wound closure could be due to the bactericidal effects of bAgNPs in the wound area that restore tissue integrity and lead to the repairing of damaged sites (Chatterjee, Chakraborty, & Basu, 2014). Tian et al. (2007) demonstrated the potential role of AgNPs in wound healing in animal model and showed that quick healing and better wound appearance took place in a dose-dependent manner (Beddy, Watson, Fitzpatrick, & O'connell, 2004; Tian et al., 2007). However, treatment with bAgNPs gel cured the wound completely with negligible scar and significant rubor loss. AgNPs also stimulate the differentiation of fibroblasts into myofibroblasts and thus induce wound contraction (Gunasekaran, Nigusse, & Dhanaraju, 2011). Similarly, Lee et al. investigated the effects of AgNPs during wound healing on dermal contraction and epidermal re-epithelialization, and suggested that AgNPs might increase the speed of wound closure (Liu et al., 2010). This property has been understood with the stimulation of keratinocyte reproduction and migration (Liu et al., 2010).

4.3.7 Antileishmanial (Antiparasitic) Activity

Leishmaniasis is caused by the protozoan *Leishmania* parasites and has been characterized as a category-1 disease by WHO (De Vries, Reedijk, & Schallig, 2015). The most common form of leishmaniasis is cutaneous that causes skin sores. On the other hand, visceral leishmaniasis affects several internal organs including spleen, and liver. However, the scarcity of antileishmanial drugs, their high cost, as well as the resistance to the existing drugs have made the situation vulnerable. To overcome this situation, plant based drugs could open a new avenue for the treatment of leishmaniasis.

Recently, it has been reported that bAgNPs can successfully kill the protozoan Leishmania. The possible mechanisms of antileishmanial activity of bAgNPs could be: (1) generation of reactive oxygen species; (2) arresting of cell cycle at G0/G1 phase; and (3) inhibition of trypanothione/trypanothione reductase system. The proposed mechanisms of antileishmanial activity exhibited by bAgNPs are shown in Fig. 4.5 (Ovais et al., 2017). Biogenic silver nanoparticles (bAgNPs) releases Ag^+ ions that act as nonenzymatic source for ROS generation. Cell cycle arrest in G0/G1 phase eventually leads to the inhibition of leishmanial proliferation. In addition, the

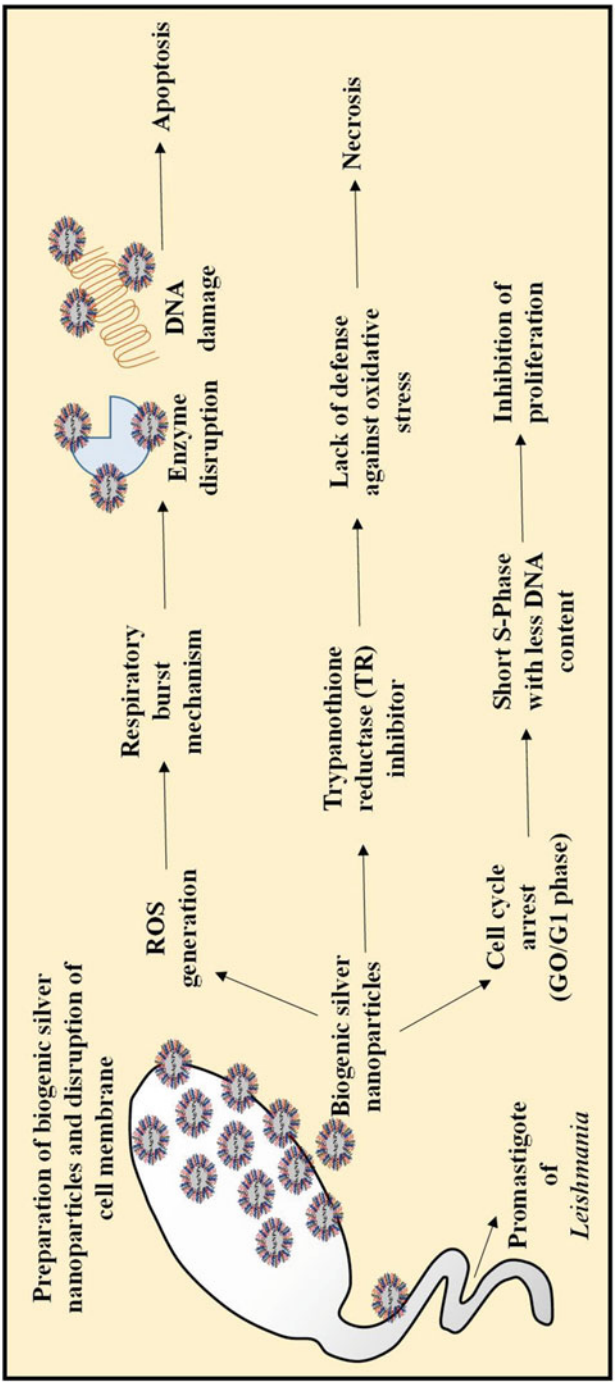

Fig. 4.5 The proposed mechanism of leishmanicidal effect of biogenic silver nanoparticles

inhibition of Trypanothione/trypanothione reductase system is also a proposed mechanism of leishmanial killing. Biogenic AgNPs inhibit the intracellular nonprotein thiols that prevent the emergence of drug resistant protozoan Leishmania (Ovais et al., 2017). The antileishmanial activity of bAgNPs synthesized using various plant extracts have been studied extensively. For example, the antileishmanial activity of bAgNPs synthesized using the dried and powdered material of a Chinese herb, *Isatis tinctoria*, was investigated against *Leishmania tropica* (Ahmad et al., 2016). Therefore, biologically synthesized silver nanoparticle could be a landmark in disease management of Leishmaniasis (Roy et al., 2019).

4.4 Application of Biogenic Silver Nanoparticles in Medical Textiles

The biogenic silver nanoparticles (bAgNPs) was synthesized using *Azadirachta indica* and *Amaranthus dubius* leaf extracts and were used to coat cotton cloth and moisture pad samples that exhibited excellent resistance to *Corynebacterium* (a sweat bacterium) (Firdhouse & Lalitha, 2015). Furthermore, bAgNPs coated gauze cloth discs showed antimicrobial activity against *Pseudomonas aeruginosa* (Firdhouse & Lalitha, 2015). The incorporation of bAgNPs synthesized from *Azadirachta indica* into the cotton cloth provides antibacterial activity against *E. coli* (Tripathi, Chandrasekaran, Raichur, & Mukherjee, 2009). Recently, bAgNPs are being commercially used in textile industries either through incorporating into fiber or coated with fiber. For example, bAgNPs are incorporated in T shirt, sporting clothes, undergarments, and socks etc. (Verma & Maheshwari, 2019). Biogenic AgNPs was applied to cotton fabrics that impart antibacterial activity to cotton fabric against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Fouda et al., 2017).

The biogenic AgNPs is extensively used in medical devices and implants and can also be used in topical ointments and creams used to prevent wounds, burns and infections (Becker, 1999). In addition, bAgNPs is also incorporated to consumer products including colloidal silver gel and silver-embedded fabrics that are used in sporting equipments (Rupp et al., 2004). Biogenic silver nanoparticles coated biomedical devices, implants (Furno et al., 2004), textile fibers (Durán, Marcato, De Souza, Alves, & Esposito, 2007) are employed for the treatment of wounds or burns and glass windows and other surfaces to maintain sanitization and hygienic conditions. The plastic catheters are coated with bAgNPs. Moreover, the researchers have developed a method of coating that yielded a thin (∼100 nm) layer of bAgNPs on the surface of the catheters. The bAgNPs coated catheters are highly biocompatible as they are nontoxic and provide sustained release of silver ions at the implantation site (Rupp et al., 2004). The risk of infection decreases many folds for these catheters because of their excellent in vitro antimicrobial potential and inhibition of biofilm formation due to the presence of *Staphylococcus aureus*, *coagulase-negative staphylococci*, *Escherichia coli*, *Enterococcus*, *Pseudomonas aeruginosa* and *Candida albicans* (Roe, Karandikar, Bonn-Savage, Gibbins, & Roullet, 2008). Biogenic

AgNPs with additive poly (methyl methacrylate) (PMMA) has been used as a bone cement because of its antibacterial potential due to presence of bAgNPs (Alt et al., 2004). It has been confirmed that nano silver-PMMA bone cement decreases the emergence of antibacterial resistance because of its versatile mode of action, and also known for its antibacterial propensity as well as low cytotoxicity (Premkumar, Lee, & Geckeler, 2010). These bone cements are effective antibacterial agents against methicillin-resistant *S. epidermidis* and *S. aureus* and showed retarded biofilm growth (Alt et al., 2004). The biocompatibility of these bone cement was investigated using human osteoblast or mouse fibroblast cells and found very good biocompatibility (Alt et al., 2004). The bAgNPs are widely used in medical and functional textiles due to their antibacterial propensities (e.g., antibacterial fabrics used to prevent infection or deodorizing) (Mantovani & Zappelli, 2009).

4.5 Biocompatibility of Biogenic Silver Nanoparticles

The biocompatibility of bAgNPs needs to be thoroughly investigated for their clinical as well as commercial applications. When bAgNPs are incorporated in medicine and as food supplements, it requires a detailed investigation on the possible cellular damages. Hence, noncancerous cell lines are used to investigate any side effects before they are recommended as safe. The biocompatibility of bAgNPs could be explained because of: (1) conjugation of the active anticancer agents of plant extracts with the biogenic AgNPs leading to near zero cytotoxicity, and (2) no release of the active anticancer agents of plant extracts from the nanoparticles once they are conjugated (Patra et al., 2015). The toxicity of silver nanoparticles is reduced by synthesizing them through biological approaches. The main reason of the minimal cytotoxicity of biogenic AgNPs is the presence of biocompatible phytoconstituents as capping agents (Roy et al., 2019). Although the primary role of the capping agents is to stabilize the nanoparticles and to prevent agglomeration, the inherent biocompatibility of coated phytoconstituents makes the bAgNPs suitable for different medicinal applications (Roy et al., 2019). Regarding the mechanism of antibacterial action, biogenic AgNPs has strong ability to reduce the level of adenosine triphosphate in the cell which ultimately brings about the mitochondria damage and stimulates reactive oxygen species (ROS) formation (Nayak, Ashe, Rauta, Kumari, & Nayak, 2016). Therefore, it is imperative to perform the comprehensive investigation on the safety and biocompatibility of biogenic silver nanoparticles. Recent study suggests that bAgNPs are stabilized by different polymers to reduce their cytotoxicity and have been investigated in vitro against various cell lines including mouse skin fibroblasts (L929), human hepatocarcinoma cells (HepG2), and mouse monocyte macrophages (J774A1). Furthermore, bioactive polymer coated bAgNPs are hemocompatible in nature at a concentration of 1.5 ppm. It is well-known that materials with less than 5% hemolytic activity are considered as hemocompatible (Lin, Lin, Dong, & Hsu, 2012). Several studies also suggested that bioactive polymer coating on the silver nanoparticle (AgNPs) reduces their cytotoxicity (Roy et al., 2019).

Since the synthesis of biogenic AgNPs is carried out by reducing $AgNO_3$ in the presence of phytoconstituents (i.e., proteins, carbohydrates, and phenolic compounds) of plant extracts, they are highly biocompatible. For example, bAgNPs derived from the aqueous extract of dried *Ficus carica* fruits was used to perform thorough toxicity study on Swiss albino female rats. A 2 g/kg single dose was administrated to the rats and were periodically monitored for 14 days for various physiological changes. It showed normal fiber integrity, hepatic veins and intact neurons when histopathological studies was performed on the brain, heart, liver and kidney and no symptoms of necropsy, infarcts, inflammation, edema as well as hemorrhage was observed, thus proving the biocompatibility of the biosynthesized bAgNPs (Jacob, Prasad, Sivasankar, & Muralidharan, 2017). The aqueous leaf extract of *Spinacia oleracea* was used to synthesize bAgNPs and the phytochemicals act as capping agents. In this study, a zebrafish embryo toxicity test was executed to judge the compatibility of bAgNPs to normal cells. The mortality rate was determined and the possible malformations on embryos were investigated under stereomicroscopy after 96 h of bAgNPs treatment. The bAgNPs exhibited complete mortality at 3 μg/mL and tail malformation with yolk sac edema was noted at 2 μg/mL (Jacob et al., 2017). In addition, phytoconstituents also showed similar results of toxicity on human cells. The bAgNPs synthesized using *Croton bonplandianum* leaves extract at its MIC dosage showed only 0.02% hemolysis (Beg et al., 2017). The aqueous leaf extract of *Azadirachta indica* proved successful in mediating the synthesis of bAgNPs to assess any probable damage on the erythrocytes by the nano particles. The erythrocyte aggregation assay was carried out which revealed that there was no toxic effect on the red blood cells (Kummara, Patil, & Uriah, 2016). The bAgNPs synthesized using the aqueous leaf extract of *Butea monosperma* were used to study any impending damage inflicted by Human umbilical vein endothelial cells (HUVEC) and their transformed version cell lines (i.e., ECV-304). Cell viability using MTT assay proved that the biosynthesized bAgNPs were fully biocompatible (Patra et al., 2015). Biogenic silver nanoparticles synthesized from *Anethum graveolens* leaf extract were also biocompatible in nature (Kalangi et al., 2016). Aqueous extract of *Rosa damascena* was used to synthesis bAgNPs of 15–27 nm size also exhibited biocompatibility against erythrocytes (Venkatesan, Subramanian, Tumala, & Vellaichamy, 2014). The nontoxic nature was confirmed when the cell viability of normal peripheral lymphocytes exhibited an amazing 117% and 109%, after a 6 h treatment with 10 and 50 μg/mL, respectively, of bAgNPs synthesized using the aqueous leaf extract of *Albizia adianthifolia* (Gengan, Anand, Phulukdaree, Chuturgoon, & Biointerfaces, 2013).

In another study, bAgNPs was synthesized from aqueous extract of *Salacia chinensis* bark offer full biocompatibility when treated against normal human fibroblasts and blood erythrocytes (Jadhav et al., 2018). The aqueous biogenic silver nanoparticles (Aq-bAgNPs) and ethanolic biogenic silver nanoparticles (Et-bAgNPs) were synthesized using aqueous and ethanolic extracts of *Andrographis paniculata* stem, respectively, as reducing agents. The bAgNPs showed excellent hemocompatibility against human as well as rat red blood cells. Furthermore, there was no significant toxicity observed when the levels of rat serum

ALT, AST, γ-GT (i.e., liver function biomarkers), and creatinine (i.e., kidney function biomarker) were determined (Hossain et al., 2019). When the epithelial cell line of humans, A549, was used for cell viability tests, there was no change in the integrity of the cell membranes after treating them with the bAgNPs prepared using the aqueous extract of garlic cloves and it was proved nontoxic (Ahamed, Khan, Siddiqui, Alsalhi, & Alrokayan, 2011).

Similar results were obtained for the bAgNPs synthesized from tea (rich in polyphenols) when the biocompatibility of bAgNPs was tested with aneuploid keratinocyte cell line (i.e., HaCaT) derived from humans (Moulton et al., 2010). Thus, the biocompatibility of green synthesized silver nanoparticles suggests its possible use in various biomedical applications (Roy et al., 2019).

4.6 Challenges and Future Prospects

A wide variety of biogenic AgNPs can be developed for various future medicinal applications including drug delivery, biomarkers, and detection and treatment of certain diseases. They can be used as a cheap and broad spectrum antimicrobial agents to protect plant crops as well as human infections. Biological source-mediated nanosynthesis has proven to be more economically scalable than physicochemical approaches. Green processes involving plant materials, and microbial cells used in the synthesis of nanoparticles are typically one-pot reactions without the use of external surfactants, capping agents and/or templates. Thus, green synthesized silver nanoparticles provide significant aspects of nanotechnology.

The future of bAgNPs seems very promising. However, it is not clear what would be the long-term impact of nanoparticles on human health and plant crops. In several studies, it has been reported that bAgNPs prevent the growth of nitrifying bacteria and, thereby, inhibit the absorption of biological nitrogen.

Conventional AgNPs generation methods require hazardous chemicals (e.g., borohydrides or hydrazine as reduction agents) and produce hazardous by-products. On the other hand, the biggest challenge in green technology is the identification of biomolecules present in the biological sources that are responsible for rapid and single-step production of nanoparticles. Furthermore, their potential undesirable effect is a major challenge for the biomedical applications of AgNPs, as AgNPs possess some toxicological effects that restrict their practical applications.

Various natural products such as extracts from various plants, microbial metabolites have been used as capping as well as reducing agents in the green synthesis processes. The advancements of genetic engineering techniques have enabled the modifications of plant and microbial genomes which, in turn, paving the way for the in vitro generation of particular plant parts containing desired phytoconstituents for the synthesis of better quality and controlled bigenic nanoparticles.

4.7 Conclusions

The application of natural products for green synthesis of nanobiomaterials is a new and promising field of nanobiotechnology. A growing awareness about green chemistry and the use of green route for the synthesis of metal nanoparticles lead to the development of eco-friendly techniques. AgNPs are the most widely used nanomaterials in the field of biomedical sciences due to their antimicrobial propensities. The synthesis of silver nanoparticles using natural resources is sustainable, energy-efficient, cost-effective, eco-friendly, and provide biocompatible and safer products. Huge natural treasure is already present on the earth as the phytoconstituents present in plant kingdom. The use of natural resources for the synthetic purposes may have an immense impact in the decades to come. There should be extensive biocompatibility investigation of bAgNPs in vivo prior to any clinical applications. This would pave the way for their clinical applications on human and animal health. Therefore, the biogenic approach would have a major direct impact on the further advancement of biotechnology.

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Translational Studies of Nanofibers-Based Scaffold for Skin and Bone Tissue Regeneration

5

Unnati Modi, Dhaval Kedaria, Bindiya Dhimmar, and Rajesh Vasita

Abstract

The area regenerative medicine targets to recreate, repair or replace the damaged body part into functional human tissue. Tissue engineering is a part of regenerative medicine that utilizes the principles and methods of engineering for the development of biological substitutes that can restore or improve the function of damaged tissues. It involves scaffolds combined with cells, growth factors or suitable biochemical signals promoting the growth and regeneration of damaged tissues and organs. Scaffolds are three-dimensional structure that mimics the native extracellular matrix (ECM) properties and provides structural support to the cells to regenerate. Several approaches have been developed for the fabrication of natural or synthetic polymer-based 3D scaffolds. Among them, nanofibers have been evaluated as promising tissue engineering scaffolds since they mimic the nanoscale properties of the native extracellular matrix and has high surface area to volume ratio. These features favors cell adhesion, proliferation, and differentiation which are highly desired for tissue engineering applications. This chapter summarizes the nanofibers' innovative approaches used to mimic ECM properties and their potential applications for the skin and bone tissue engineering. Also, the successful translational studies of nanofibers for *in vivo* skin and bone regeneration have been elaborated in detail.

Keywords

Regenerative medicine · Bone tissue engineering · Nanofibers · Skin tissue engineering · Electrospinning

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5.1 Regenerative Medicine (RM)

5.1.1 Definition

Tissues and organs injured by trauma, disease, and aging necessitate developing the therapies which can regenerate tissues and decrease the reliance on transplantations. 'Regenerative medicine' is a branch of translational research in tissue engineering and molecular biology which deals with the "process of replacing, engineering or regenerating human cells, tissues or organs to restore or establish normal function" (Mason and Dunnill 2008). Regenerative medicine has the potential to regenerate, augment or replace damaged tissues or organ. (Mao and Mooney 2015). It includes the *in vitro* growing of tissue and organs and then implanted into the body when it cannot heal itself (Prasad et al. 2017).

5.1.2 Need and Role of RM

The aim of regenerative medicine is to create the functional human tissue to either repair or to replace the damaged body part. It gives the possibilities to generate bioengineer human tissue *in vitro* in the future by scientist that may allow the identification of novel medicines and has the potential for cell-based therapies (Hay 2011). It has the ability to exclude the issue of organ transplant rejection by regenerating organs from the patient's own tissue or cells (Prasad et al. 2017). The main objective of regenerative medicine is to develop the biomaterials which release the bioactive factors for promoting appropriate tissue formation (Fisher and Mauck 2013). Cellular therapies remain a large focus, and the efficiency of various cell types have been assessed *in vitro*. Moreover, significant efforts in regenerative medicine and biology have focused on the developmental origins of stem and progenitor cells, and on elucidating how they persist (or don't) in adult organisms (Mohammadian et al. 2017). Figure 5.1 shows the major approaches of the regenerative medicine.

5.1.3 Tissue Engineering as a Method of RM

Tissue engineering is one component in the field of regenerative medicine (Greenwood et al. 2006; Mason and Dunnill 2008; Vacanti 2010) Stem cells science, gene therapy, soluble molecules, and reprogramming of cell and tissue types are each part of the interface between tissue engineering and regenerative medicine (Shafiee and Atala 2017). Tissue engineering is an integration of engineering principles and biological facts. This field has gifted many exciting and innovative medical devices which works to enhance, restore or replace the damaged tissue or organ (Yen and Yelick 2011). Tissue engineering reduces the critical shortage of donor organs by *in vitro* fabrication of functional biological scaffolds (Shafiee and Atala 2017). It includes scaffolds integrated with cells and growth factors or suitable

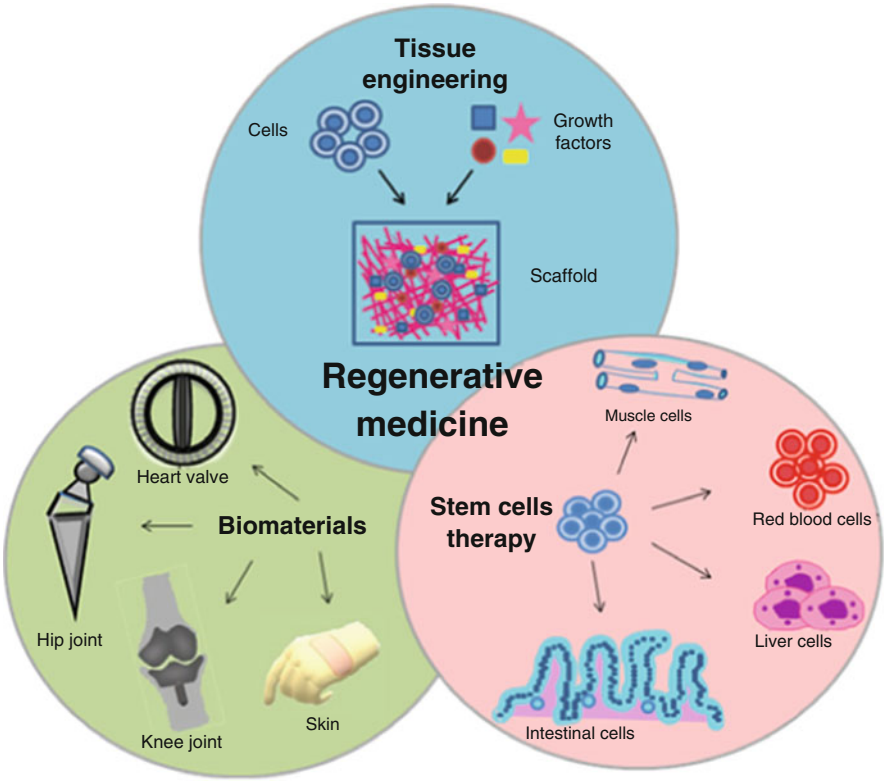

Fig. 5.1 Three approaches of regenerative medicine

biochemical signals which enhance the growth and regeneration of new tissues and organs.

5.2 Scaffolding for RM

5.2.1 Role of the Scaffold in Tissue Engineering

Three-dimensional network or structure that can be produced by natural, synthetic polymers and purely biological molecules such as collagen, elastin, hyaluronic acid, and other molecules of extracellular matrix (ECM) known as Scaffold (Bacakova et al. 2014). It mimics both chemical composition and physical structure of native ECM. At the defect site, it provides temporary mechanical integrity until the damaged tissue is functionally repaired or regenerated (O'Brien 2011; Zhou and Lee 2011). To achieve the functionality, there is a number of characteristics which are important when designing or the scaffold for tissue engineering:

Table 5.1 Types of Scaffolds—Fabrication techniques and its unique properties

Type of Scaffold	Method of fabrication	Unique properties
Hydrogel	Phase separation, freeze drying, in situ polymerization, micromolding, photolithography, microfluidics, emulsification, salt leaching, gas foaming, porogen leaching method	Macroporous interconnected framework, biomimicking bulk properties, injectable
Acellular scaffold	Decellularisation	Unique anatomic structure like native ECM
Fibrous scaffold	Wet and melt electrospinning, self-assembly, phase separation	Micro-nano scale surface topography, ECM mimicking geometry, high porosity and surface area to volume ratio
3D printed scaffold	Stereolithography, inkjet printing process, computer-aided design (CAD) data manipulation techniques, organ printing	Customized geometry with high precision, cell encapsulated printing
Ceramic scaffold	Sponge replication method, simple calcium phosphate coating method	Interconnected porous structure, high mechanical strength, and load-bearing capacity

1. *Biocompatibility*: Biocompatibility is the most important criterion of any scaffold for tissue engineering. Before the production of the new matrix, Cells must be able to adhere and migrate onto the surface and eventually through the scaffold and started to proliferate. After implantation, the scaffold must elicit a negligible immune reaction in order to prevent it from causing an inflammatory response which reduces healing or causes rejection by the body.
2. *Biodegradability*: The aim of tissue engineering is to replace the implanted scaffold after some time by body's own cells and the extracellular matrix produced by them. Therefore the scaffold must be biodegradable.
3. *Mechanical properties*: The mechanical strength of the scaffold in such a way that it can withstand with the biological implantation site and it must be able to allow surgical handling during implantation.
4. *Scaffold architecture*: The construction of scaffold is also an essential criterion in tissue engineering. The scaffold should have an interconnected porous structure, suitable pore size, high porosity to allow cells infiltration, nutrient diffusion, and ECM production by cells within the scaffold (O'Brien 2011).

A unique structured scaffold with desirable properties is required for every tissue (Carletti et al. 2011). To fabricate the scaffold, different types of (Natural and Synthetic) materials have been used. Biomaterials based 3D scaffolds have been fabricated by using different techniques (Atala and Lanza 2002). Most commonly used scaffolds, their method of fabrication, and unique properties are listed in Table 5.1 and SEM images of different types of scaffolds are shown in Fig. 5.2.

The main objective of tissue engineering is to fabricate the scaffold which influences the cells of tissue for remodeling (Nigam and Mahanta 2014). All of the

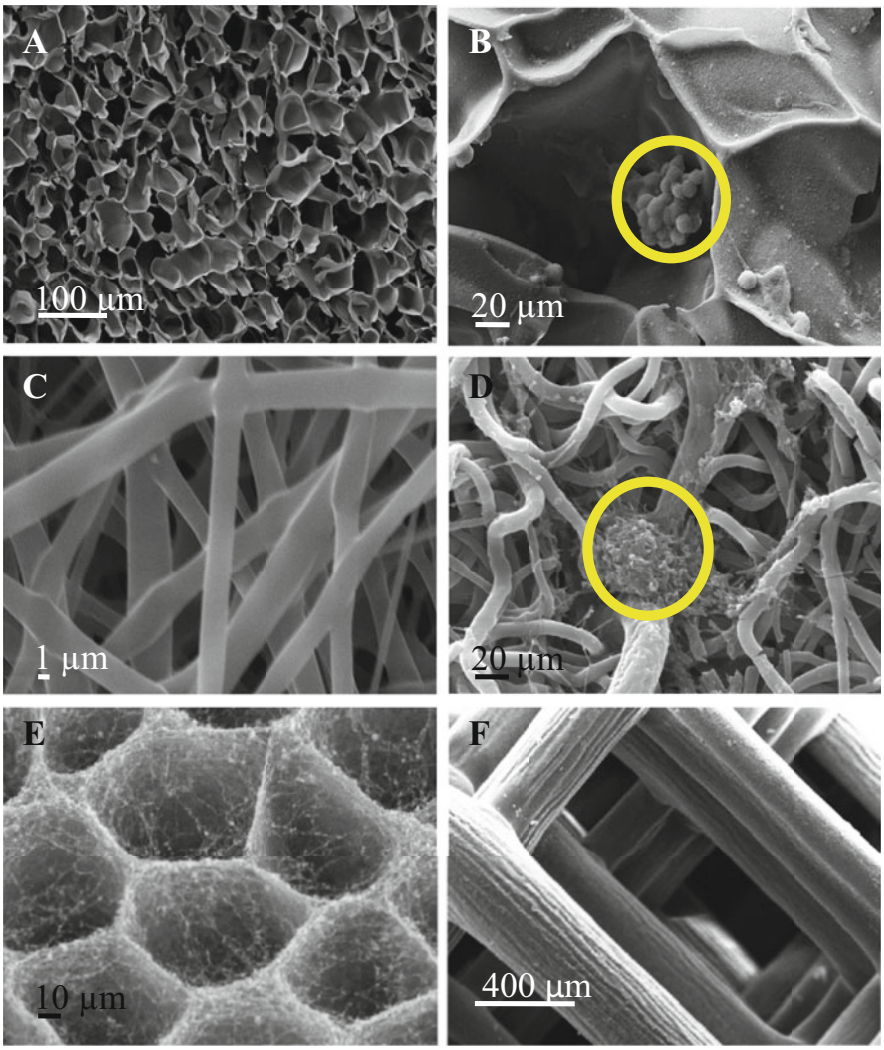

Fig. 5.2 SEM images of different types of scaffolds. (**a**) Hydrogel, (**b**) Tagged portion represents A549 cells grown on hydrogel, (**c**) Nanofibers, (**d**) Tagged portion 3T3 fibroblast cells grown on nanofibers, (**e**) Honeycomb-patterned nanofibrous structure (Yan et al. 2011) re-printed with the permission of American Chemical Society, Copyright © 2011 (**f**) 3D printed scaffold (Cutting et al. 2016) re-printed with the permission of frontiers

above mentioned scaffolds have their own unique properties while a tissue engineered scaffold require optimum porosity, mechanical strength with a unique anatomic structure like native ECM. Therefore, the fibers produced by an electrospinning technique is considered as the most promising scaffold in tissue engineering by reason of its nanoscale properties of native ECM (Pham et al. 2006).

It has several remarkable characteristics such as porosity, higher surface area to volume ratio, remarkable mechanical properties, and provides flexibility to functionalize the surface. Due to these properties of nanofibers, it serves as an ideal candidate for many important applications such as tissue engineering, and controlled delivery of drugs, protein, and DNA. (Huang et al. 2003; Vasita and Katti 2006).

5.2.2 Nanofibrous Scaffolds

In tissue engineering, to fabricate the scaffold which mimics the native architecture of tissue is very challenging. The production of the nanofibrous scaffold can potentially meet this challenge (Vasita and Katti 2006). Due to its topographical features, it allows cell adhesion, proliferation, and differentiation which is essential properties for tissue engineering applications. Therefore, nanofibrous scaffolds have been widely explored for various tissue engineering applications (such as bone, skeletal muscle, skin) and controlled delivery of Drugs (Kenawy et al. 2002), DNA (Fang and Reneker 1997; Luu et al. 2003) and proteins (Zeng et al. 2005) to the desired biological site.

Nanofibers have been fabricated by using various techniques such as phase separation, self-assembly, and Electro-spinning. Among all of these techniques, the most extensively studied technique for tissue engineering application is Electrospinning. (Vasita and Katti 2006).

5.2.3 Techniques for Nanofibers Fabrication

5.2.3.1 Phase Separation

Based on thermally induced liquid-liquid phase separation (Ma and Zhang 1999), the nanofibrous foam is formed which involves the following steps (Zhang and Ma 2000, 2001):

1. Polymer dissolution
2. Liquid-liquid phase separation
3. Gelation of polymer
4. Extraction of solvent from the gel with water
5. Freezing of the extracted polymer from the gel
6. Freeze-drying under vacuum

The morphology of nanofibrous scaffold can be controlled by altering some parameters such as heat treatment, type of polymers and type of solvent (Zhang and Ma 2000).

5.2.3.2 Self-Assembly

Self-assembly is the process which involves the organization of smaller components to form larger, ordered aggregates without the outward control (Whitesides and Boncheva 2002). It is defined as "the noncovalent interaction of two or more molecular subunits to form an aggregate whose novel structure and properties are determined by the nature and positioning of the individual components" (Tecilla et al. 1990). In the biological system, there are various examples present such as Cell membrane (Ng et al. 2004) (contain Hydrophilic head group and Hydrophobic tail group), the process of protein folding. The process of self-assembly is appropriate for component size ranging from molecular to macroscopic level. Generally, the process of self-assembly is accomplished by non-covalent interactions such as Hydrogen bonding, and Van der Waals forces (Whitesides et al. 1991).

The environment surrounding the cells, is identified by cell's receptors like the ECM molecules such as Collagen, Integrin, and Fibronectin. To mimic the native ECM, nanofibers have been produced based on the peptide-amphiphile self-assembly system (Berndt et al. 1995). Peptide-amphiphile contain hydrophobic lipid chain and the hydrophilic head group made up of charged amino acids (Hamley 2011). Generally, a head group of PA made up of a peptide sequence which is similar to native ECM molecules (like collagen). The advantages of the self-assembled nanofiber are that it can be used as a bio-resorbable scaffold because this scaffolds constitutes the natural L-amino acids and it gives no detectable immune response.

5.2.3.3 Electrospinning

To fabricate the nonwoven nano-micro fibrous mesh, the most extensively used technique called Electrospinning. In electrospinning technique, the syringe has been filled with a polymeric solution and it is attached to the syringe pump. The positive electrode attached to the needle and the negative electrode attached to the collector plate and the high voltage is applied to the polymer solution through the needle. The specific flow rate is applied by the pump and the electric field is generated by applying the high voltage power. Due to this high voltage, the polymer solution is stretched towards the grounded collector.

When a sufficiently high voltage is applied to the liquid droplet of polymer, the body of the liquid becomes charged and electrostatic repulsion counteracts the surface tension and the shape of the droplet change from spherical to a conical shape which is known as "Taylor cone" (Doshi and Reneker 1995; Yarin et al. 2001). Now due to sufficient molecular cohesion of the liquid, the stream of liquid does not break up but the charged liquid jet is formed. After that, the jet is elongated by the whipping process which is caused by electrostatic repulsion until the (Shin et al. 2001a, 2001b) fibers are finally deposited on the grounded collector (Zeleny 1914; Reneker et al. 2000; Shin et al. 2001a, 2001b; Frenot and Chronakis 2003). Generally, there are mainly three forces involved in this process (1) Surface tension, (2) Electrostatic repulsive force derived from the electrically charged polymer droplet, and (3) the Viscoelastic force coming from the polymer.

The entire process of electrospinning is controlled by three types of parameters: (1) System parameters such as Viscosity and concentration of the solution, molecular

weight of the polymer, properties of the solvent, surface tension and conductivity. (2) Processing parameters such as applied voltage, flow rate, needle gauge, the distance between the tip and collector (3) Atmospheric parameters such as temperature and humidity (Li and Wang 2013).

In this chapter, regenerative medicine for skin and bone has been discussed in detail, considering the approach of tissue engineering with the help of electrospun nanofiber based medical devices. For better understanding of tissue regeneration, the surrounding environment where the device is implanted, the anatomy and physiology of skin and bone has been explained. The chapter contains brief information about the skin and bone injury healing process, and the conventional approaches used for treating them. Further, we have explained why there is a need for regenerative medicine, the role of electrospun nanofiber based medical devices and related *in vivo* studies. The chapter further states the electrospun nanofiber based medical devices as a potential regenerative medicine to be used translationally in future.

5.3 Skin Tissue Engineering

5.3.1 Human Skin: Anatomy and Physiology

The Skin, key part of the integumentary system is the largest organ. It is a protective barrier of our body, the first line of defense of our innate immunity against the foreign invaders from the surrounding environment and a habitat of a diverse population of microbes (Findley and Grice 2014). It protects against a harmful Ultraviolet (UV) radiation, chemical, mechanical and pathogenic microbes. Also, it has a major role in fluid balance and body temperature maintenance in humans (Biedermann et al. 2013; Böttcher-Haberzeth et al. 2010; Groeber et al. 2011).

The structure of skin constitutes three layers; (1) Avascular epidermis (upper layer), (2) Dermis highly supplied with blood vessels (middle layer) and (3) Hypodermis (lower layer) (Supp and Boyce 2005; Takeo et al. 2015). Epidermis layer acts as a barrier against different chemical, mechanical agents, and pathogens. It also maintains the balance of skin fluid. This layer is constituted of (1) Melanocytes that play a key role in pigmentation, (2) Keratinocytes arranged in four strata comprising strata basale, spinosum, granulosum, and corneum (3) Langerhans' cells, the dendritic cells family member that is involved in the primary immune response by skin and (4) Basal cells present in the deep layer. These cells have high proliferation capability and aids in the formation of stratified epithelium. Thus, skin functions as an efficient protective layer on the body surface (Badylak 2002; Metcalfe and Ferguson 2007; Takeo et al. 2015).

The dermis is a thick layer of epidermis consisting mainly fibroblasts (Varkey et al. 2015). By secreting and remodeling of the extracellular matrix (ECM) components, fibroblasts play a vital role in wound healing. The ECM constitutes of Glycosaminoglycan (GAGs), collagen, elastin and dermal cells like smooth muscle cells, fibroblasts, mast cells, and endothelial cells are present in the nearby vicinity. Several proteins involved in ECM provides structural support and controls

the growth of cells by functioning as growth factors. Thus, ECM behaves like a 3D scaffold (Chua et al. 2016; Supp and Boyce 2005; Varkey et al. 2015). The key factor for the mechanical and elastic property of the skin is the fibrous nature of the ECM and has a good vascular system for skin nourishment. The integrity of the human skin is a result of connective tissue protein linked with GAGs (Badylak 2002; Kalluri 2003; Varkey et al. 2015). Collagen is the vital protein of ECM. The crosslinked collagen and elastin have a fundamental role in providing elasticity for dermis (Supp and Boyce 2005). The hemidesmosomes are formed by the dermal fibroblasts secreted Collagen types IV and VII along with laminin. Thus, hemidesmosomes mediate fibril to anchor and in turn helping keratinocytes to attach to the dermis. A hydrophilic space is formed by Chondroitin sulfate and HA which is negatively charged. It provides dermis, capacity to handle stress and mechanical disturbances (MacNeil 2008; Shah and Amini-Nik 2017; Tracy et al. 2016). Another abundant protein in the ECM is Fibronectin and laminin of basement membrane that provides adhesion to the cells due to its structure (Miyamoto et al. 1998; Schwarzbauer 1991). Also, there are receptors for touch, pain, and temperature in the dermis. Moreover, glands (hair follicles, sweat, and sebaceous glands) responsible for skin lubrication and temperature maintenance, pass through epidermal and dermal layers to reach the surface. A layer of keratinocytes coats the sweat gland and plays a crucial function in epidermal healing (Breitkreutz et al. 2009; Sorrell et al. 2008; Tracy et al. 2016).

Hypodermis (subcutis), the skin deepest layer consists of adipocytes which serve a significant role in insulation, thermoregulation, mechanical properties, and energy supply. Consequently, these three layers of skin coordinate and contribute vitally to maintain the skin tissue homeostasis and integrity.

5.3.2 Wound and its Healing Process

Wounds are categorized mainly on the bases of wound depth as (1) First degree or superficial (affects epidermis) (2) Second degree or partial thickness (damage in both epidermis and dermis) (3) Third degree or full thickness (injury up to dermis and

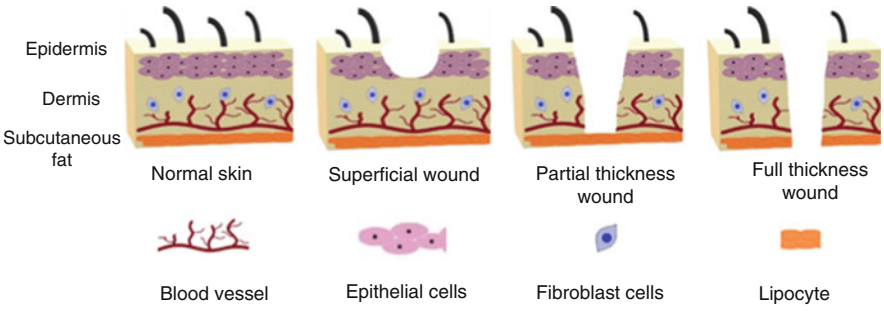

Fig. 5.3 Wound classification according to their depth

damages subcutaneous fat lining). Any skin defect can be classified in these categories especially in burn injuries. Wounds classified according to the depth of skin tissue damage has been shown in Fig. 5.3. Deep wounds including hypodermal and dermal injuries can further cause bacterial infections, fluid imbalance, drop in thermoregulation and may also cause impairment in some cases (Damanhuri et al. 2011; Dixit et al. 2017; Kahn et al. 2011; Varkey et al. 2015). Delayed and inappropriate care of trauma caused acute wounds may lead to scar formation (Carter et al. 2014). Acute wounds generally go through systematic healing steps consisting of inflammation, proliferation, tissue remodeling and scar formation within an anticipated period (Demidova-Rice et al. 2012). Even though autologous transplantation has disadvantages, it is still a widely used treatment for serious wounds. Chronic wounds are the wounds that don't heal in sequential stages and takes more than 3 months to heal. Intense pain, the delayed healing process, high infection rate, inadequate autologous skin to wrap up the large surface area wounds, and graft failure, it seems less effective strategy and has become a challenge for specialists and patients (Demidova-Rice et al. 2012; Järbrink et al. 2017). Current progress in the wound treatment can aid in the fluid balance caused by wound formation, infection control at the wound site and effective fast healing.

After the injury, wound healing involves a cascade of the signaling pathway. The process has been divided into four different stages as (1) Damage limitation, (2) Inflammation, (3) Proliferation and (4) Maturation or remodeling (Biedermann et al. 2013; Liu et al. 2017; Midwood et al. 2004; Wynn 2008). The first stage i.e., damage limitation step involves blood clot formation. This forms short-term blockage which limits bleeding, fluid loss, and protects from further bacterial infection. Along with the process of blood clotting at the wound site, the signal sensitive cells also rush there (Shakespeare 2001; Vig et al. 2017). In the next phase, signs of inflammation like local swelling, redness, and edema are observed which are aided by the elevated blood flow and increased vascular permeability. Additionally, temporary ECM is formed, and it contains fibrinogen, fibrin, and fibronectin. Secretion of vasoactive compounds like histamine leads to diapedesis of leucocyte which then activates the complement system (Rhett et al. 2008; Xue and Jackson 2015). Further, the dermal fibroblasts undergo proliferation in response to cytokines (Xue and Jackson 2015). Following the proliferative phase, the cells involved in inflammatory reactions release growth factors, which acts as a stimulus. In response to this stimulus, vascular endothelial cells and fibroblasts proliferate further. The final wound closure is accomplished by the epithelial cell proliferation at the injury boundary (Catalano et al. 2013; Shakespeare 2001; Takeo et al. 2015; Vig et al. 2017; Wynn 2008). The keratinocytes migrate from the wound border to the surface of granulation tissue beneath the blood clot. In the last phase of wound healing, mature epithelium and scar formation occur by fibroblasts. This process may take a month or more.

5.3.3 Conventional Approaches for Wound Healing

Regeneration and fibrosis are the two different mechanism through which the wound healing process proceeds. Reconstruction of tissue to the native state is the result of regeneration and functionally deteriorated skin, scar formation, overproduction of collagen and connective tissue are the results of fibrosis (Gurtner et al. 2008). Different methods are being implemented to treat wounds. One of them is autologous skin grafting which is used to treat hypodermal and deep dermal wounds, especially which are caused due to the burning of skin (Dixit et al. 2017; Vig et al. 2017). Grafts obtained from the frozen cadavers or from the live subject is known as skin allograft. It enhances angiogenesis, cytokines and growth factors' production. But it has some disadvantages like it can trigger inflammation application loci, rejection by the host immune system and transmission of viral infection (Cardinal et al. 2009; Rockwell et al. 2003; Vig et al. 2017). Another alternative used is xenograft—the grafts obtained from other species. For example, collagen obtained from other animal is applied to the lesion site which in turn stimulates wound healing. Skin substitute derived from the Porcine and Bovine source are the most generally used Xenografts (Halim et al. 2010; Nathoo et al. 2014; Shores et al. 2007; Vig et al. 2017). Such a graft has maximum chances of immune rejection. Due to various limitations and associated obstacles of above mentioned methods, need of an alternative skin graft option arose which could lead to the development of successful transplantation technique.

5.3.4 Demand for Engineered Skin Substitutes

Grafts don't exhibit satisfactory clinical result all the times (Debels et al. 2015; Munster et al. 1990). Furthermore, it requires a great extent of precautions, surgical skill, and many other resources to provide proper wound care (Shakespeare 2001). Further, Patients having diabetes mellitus or improper functioning venous valves leading to hypoxic condition, further hinder healing of the wound timely. In such cases, choosing a conventional graft approach may not be sufficient enough. Like in the case of chronic wounds, severe ulcers which take a minimum 12 weeks of time when treated with regularly used clinical treatments (Campbell and Parish 2010; González-Consuegra and Verdú 2011; Langer and Rogowski 2009). The necessity for developing skin grafts arose due to some drawbacks of the conventional methods which hindered their success in wound treatment. To provide an alternative which can overcome the limitations of the conventional method a new discipline well known as 'Tissue engineering' emerged out. It involves multiple field experts from the cell and molecular biology, biomaterial researchers, and clinicians. For example, a biocompatible carrier seeded with a layer of keratinocytes which can aid the development of appropriate microenvironment for epithelial cells and fibroblasts to heal the wound and lessen the adverse consequences of the conventional approach (Langer and Rogowski 2009; Shakespeare 2001). Such biomedical devices can be used as a graft. It helps in healing the treatment resistant wounds and helps in

regaining the native functions of the injured tissue. Various such bioengineered skin grafts have been invented. Various studies performed with bioengineered grafts have supplemented proof for them being better than the conventional grafts used for wound healing in certain aspects.

5.3.5 Wound Healing Study Models

Various models have been developed for studies related to wound. One of them is *in silico* modeling which is governed by mathematical equations formulated according to the existing knowledge about the cellular behavior. It performs a simulation of cell-cell interaction, their environment for the wound healing. *In vitro* studies involve defect mimicking wound created in monolayer or studies involving cells grown using scaffold by co-culture method. *Ex vivo* include use of harvested cutaneous tissue to which artificial wound is induced having a full or partial thickness and *in vivo* models which involve the use of either animal models or human volunteer to which a wound is created or patients actually having wound. The focus of the present chapter is, *in vivo* studies performed on the animal models in the context of wound healing. The murine model is the most economical and extensively utilized model to which generally wound is created on the tail, head, back, and ear for the various studies. Also, they are amenable to genetic manipulation. Despite the advantages, primarily wound healing is through a contraction in mice, which is remarkably unlike in humans (Grose and Werner 2004; Reid et al. 2004; Vincent et al. 2004). Pigs accurately mimic the human healing process for the wound (Sara and Ardeshir 2017). Pigs have sparse hair, skin adherent to underlying structures, and a thick dermis which imparts them structural similarity to humans (Lindblad 2008). Despite having a similarity to the human skin, the less vascular dermis is a major difference compared to human skin (Montagna and Yun 1964). Comparing with their murine counterparts, Pigs are difficult to manage and more expensive. Moreover, the ethical point of view should be kept in mind while planning to use any animal model.

5.3.6 Scaffolds for Skin Tissue Engineering

Skin regenerative substitutes include mainly scaffolds, cells, and growth factors. Scaffolds can be made up of different polymers each providing special physiochemical features. The scaffolds provide additional credit to the cell for adhesion, proliferation, and differentiation, thus performing as the 3D ECM analogs and neovascularization process (Nicholas et al. 2016). In the manufacturing of skin substitutes, three main types of scaffolds viz. synthetic, natural and composite (the combination of synthetic and natural) are used and discussed below.

The natural biomaterials have great skin regeneration potential because of their characteristics like biocompatibility, micro-inflammation, nontoxic catabolites, ability to induce tissue repair and regeneration (Da et al. 2017). Biologically existing

functional and structural ECM components such as gelatin (Ostrovidov et al. 2014), elastin, collagen (Yu et al. 2017), HA (Hyaluronic Acid), Chitosan (Hoseinpour Najar et al. 2017), fibrin, fibronectin, alginate, laminin, pullulan are common biocompatible material suitably utilized to synthesize scaffolds for tissue engineering of skin.

Synthetic materials are majorly composed of hydrocarbons. The biological characteristics of natural biopolymers are certainly not exhibited by them, but their easy production process and controllable parameters, make them advantageous for translational use in wound healing (Sheikholeslam et al. 2018). Generally used artificial biomaterials are Polyhydroxyortho Esters (POE)—Polylactic Acid (PLA) (Jouybar et al. 2017), Polyglycolic acid (PGA), and Poly Lactic-co-Glycolic Acid (PLGA) (Dwivedi et al. 2018) are subgroups of POE. Moreover, Polyethylene Glycol (PEG) (Mohammadi et al. 2017), Poly-ε-caprolactone (PCL) (Levengood et al. 2017; Sun et al. 2018), Poly-β-Hydroxybutyrate (PHB), Poly Vinyl Alcohol (PVA), Polyurethane (PU) are also widely used. Further, Self-Assembling Peptides (SAPs) consisting nano-biomaterials are designed, which comprises of a chain of amino acids combined with the scaffold. The major applications of SAPs are drug release and tissue engineering (Bradshaw et al. 2014; Kyle et al. 2009; Schneider et al. 2008; Wu et al. 2012).

Combination of synthetic and natural biomaterials together are used in which synthetic polymers provide mechanical strength and natural polymers impart biocompatibility. This has proved to mimic the native skin tissue and being an ideal skin substitute for human normal skin (Sheikholeslam et al. 2018). Varieties of polymer combinations have been developed to get desired properties such as optimum pore size, the rate of biodegradation, mechanical strength, hydrophobicity, firmness, molecular weight. Also, it can aid to achieve some other essential properties like anti-inflammatory property and growth factor release required for a scaffold to act as the best tissue engineered graft for skin injury. Some widely explore composite scaffolds are polyethylene oxide–chitosan (Bhattarai et al. 2005), PLLA–collagen (Hall Barrientos et al. 2017), PCL–collagen (Tanha et al. 2017), chitosan/PVA (Wang et al. 2017), and chitosan/collagen/PEO (Rahmani Del Bakhshayesh et al. 2018). In recent years, usage of natural extracts and blends are in demand as a main component for the composite scaffold. The scaffolds fabricated using composite polymers can be considered as the most productive for tissue engineering. Incorporating growth factors in the scaffolds can accelerate and regulate some essential mechanism like neovascularization, cell migration, and fibrosis. Growth factors like Fibroblast Growth Factor (FGF) (Sun et al. 2014), Epidermal Growth Factor (EGF) (Choi et al. 2008), Platelet-derived Growth Factor (PDGF) (Yuan et al. 2018), Vascular Endothelial Growth Factor (VEGF) (Xie et al. 2013), help in producing functionally advanced skin substitutes and efficient wound regeneration (Nicholas et al. 2016). A recent advance in the skin substitutes has evolved drastically. For example, cellular autologous skin substitutes which are mainly of two types—Cultured Skin Substitutes (CSS) and Cultured Epithelial Autograft (CEA) (Vig et al. 2017). This is a new and effective therapeutic strategy for critically burnt patients. The CEA comprises of keratinocytes which are derived by performing

biopsy from the small area of normal skin. The cells are allowed to proliferate in the laboratory to make CEA sheets delivered through the carrier system. This improves the healing mechanism and decreases the time required for wound healing (Lataillade et al. 2017). In the past few years, *in vivo* studies done using various types and composition of electrospun nanofibers as a regenerative medicine has been described in the below section.

5.3.6.1 Nanofibrous Scaffolds for Skin Tissue Engineering: *In vivo* Studies

For years, natural resources are been used in various forms due to their medicinal benefits and on the other hand, nanofibers are considered as an appropriate candidate for wound dressing because of their capabilities such as enhancing of hemostasis, cell growth, absorption of wound exudates (Hoseinpour Najar et al. 2017). Thus, utilization of herbal extract and their biologically active compounds in nanofiber synthesis is an increment in the area of nanofibers and its biological applications. Generally, wound initiate to contract at about 7 to 14 days post-injury. Quicker the wound is closed, lesser will be the pain, and it reduces the chances of infection and scar formation (Singer and Clark 1999). Nanofibrous dressing based on soy protein has been engineered using rotary jet spinning (Ahn et al. 2018) comprising of soy protein hydrolysate (SPH) and cellulose acetate (CA). There are bioactive peptides in Soy protein similar to that present in ECM. Also, it carries phytoestrogen having anti-inflammatory (Chacko et al. 2005), anti-bacterial (Ben Arfa et al. 2007), and anti-oxidant (Peñta-Ramos and Xiong 2002) features that enhances wound healing. These nanofibers accelerated epidermal thinning, re-epithelialization as well as collagen anisotropy and reduction in scar formation as observed in the *in vivo* studies on mice excisional wound. After 7 days of surgery, the scaffold treated wounds showed 72% wound closure more than compared to control wounds. In another study, a natural honey flavonoid Chrysin, having anti-inflammatory and antioxidant effects, loaded electrospun nanofiber were examined for wound healing (Mohammadi et al. 2017). Chrysin concentration used was 20% to the PCL-PEG-PCL concentration. They were loaded to the same copolymer and this scaffold was evaluated in rats. Its effect on the genes playing a crucial part in wound healing process were compared to control groups, 10 days post surgery, and the effect on the expression of the IL-6, MMP-2, MMP-8, MMP-9, TIMP-1, and TIMP-2 was increased by 4.5, 5.0, 5.5, 5.0, 1.0, and 0.8 folds respectively. Likewise, Aloe vera gel was investigated for wound healing purpose. It showed that it increased the amount of collagen in the wound as it contains vitamin C, vitamin E, accelerating the wound healing process. The gel coated electrospun PLLA nanofibrous scaffolds was used as a wound dressing for full-thickness skin defect in mice and demonstrated the accelerated wound-healing process by 74% wound coverage after 7 days compared to 36% in case of the control group (Jouybar et al. 2017). Nanofibers electrospun using Bixin (a constituent of Carotenoid pigments from Bixa Orellana L. seeds having anti-oxidant, anti-inflammatory and hypoglycemic effect) loaded PCL was explored as a controlled delivery system which showed about 80% efficient

cutaneous wound healing compared to 70% by pure PCL nanofibers after 7 days of surgery done on induced diabetic mice (Pinzón-García et al. 2017).

The amino acid L-arginine is an important substrate for the synthesis of nitric oxide (NO). There are three isoforms of nitric oxide synthase has a key function in the healing mechanism of the wound (Witte and Barbul 2002). Thus, arginine is used for functionalizing nanofibers targeted for the wound healing purpose. *In vivo* study done by using arginine modified chitosan nanofibers (Arg-Chi-NFs) done on rats showed that there was $93.8 \pm 3.1\%$ decrease in the wound area when applied with Arg-Chi-NFs gel after 9 days when compared to the wound area at day 0. Whereas, the group treated with the chitosan nanofibers with sodium alginate mixture, with arginine solution and the control showed $76.5 \pm 4.5\%$, $60.4 \pm 2.5\%$, $51.2 \pm 3.9\%$ area wound closure, respectively. The wound healing efficiency is depicted by the percent wound closure which is the measurement of the wound area (mm^2). The wound area closure is measured on the desired day post surgery and compared to the wound area on the day of surgery (Hoseinpour Najar et al. 2017). Similarly, Lignin is getting attention as a potential biomaterial, it exhibits anti-diabetic, anti-oxidant, and anti-microbial activities (Barapatre et al. 2015; Kai et al. 2016). Nanofibers were fabricated from Lignin and further its surface was altered by arginine through electrostatic interaction and gel was prepared (Arg-Lig-NF gel). This gel was applied to treat full-thickness excisional wounds created in rats. Its efficiency for wound healing was compared to Lig-NF gel and arginine solution. Nine days post surgery, the percentage area of wound healed was $93.20 \pm 3.65\%$ relative to day 0 in the group to which Arg-Lig-NF gel was applied on wound, whereas only $70.23 \pm 4.76\%$ in Lig-NF gel treated group, $57.26 \pm 4.85\%$ in arginine solution treated group, and $49.1 \pm 4.18\%$ normal saline – control group. Further, Diabetic wound healing is a slow process. One of the reasons for it is unstable functioning of hypoxia-inducible factor 1a (HIF-1a) in the hyperglycemic condition. One such study of the wound dressing developed for diabetic wound has been discussed. Nanofibers releasing Dimethyloxalylglycine (DMOG) were fabricated using co-axial electrospinning. Fibers having DMOG in the core was addressed as C-DPC, PCL/Col I mono-axial nanofibers containing DMOG or the PCL/Col I fibers alone were denoted as M-DPC and PC, respectively. Wound healing potential of these fibers was studied in rats. Seven days post surgery, the percentage of wound area closure in the C-DPC group was 66%, 59%, 57% and 33% in the M-DPC, PC and control groups, respectively. Also, after 7 days, the level of expression of the genes related to wound healing were up-regulated in human foreskin fibroblasts by Col I, Col III, a-SMA and TGF-β1 4.5, 5.0, 4.5, 4.0 folds, respectively on C-DPC nanofibers compared to PC nanofibers. The expression level of HIF-1a target genes involved in wound healing i.e., VEGF, VEGFR, HSP-90a, SCF and SDF-1a was up-regulated by 2.5, 10.0, 2.5, 5.0, and 1.5 folds respectively (Gao et al. 2018).

Additionally, there is one more beneficial application of nanofibers is the fiber alignment. The aligned nanofibers induce more elongated cellular morphology and faster cellular migration compared to random nanofibers like proved in the study carried out with dermal fibroblasts on aligned nanofibers. The cells showed elongated morphology and accelerated migration in the direction same as of the fiber

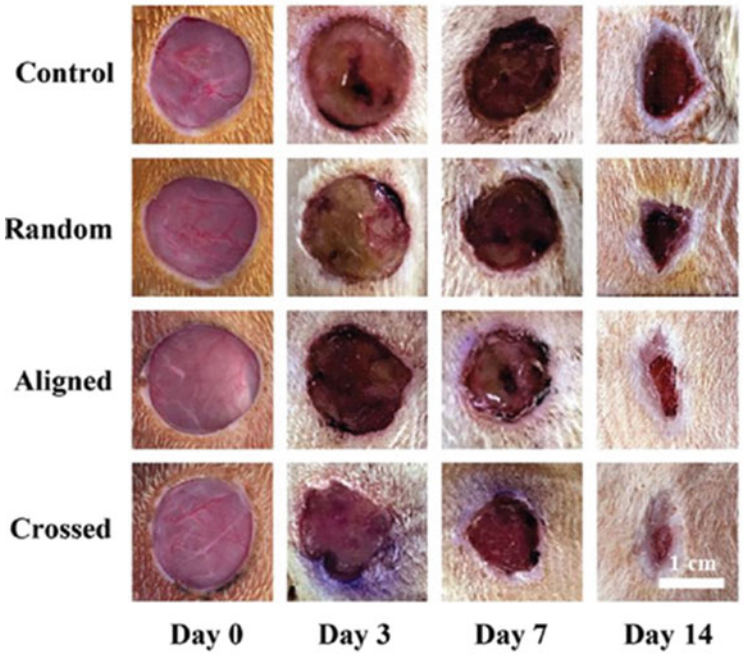

Fig. 5.4 Representative images of the full-thickness wound beds treated with different fiber spatial arrangements (random, aligned and crossed) scaffolds for 0, 3, 7 and 14 days in diabetic Rat. Reprinted with the permission from Ref. (Sun et al. 2018) copyright © The Royal Society of Chemistry

orientation. (Huang et al. 2012). Likewise, the cross-patterned PCL-Collagen I nanofibers was tested for healing full thickness wound in mice. Figure 5.4 represents the full-thickness wound beds treated with various nanofibrous scaffolds in this study. After 7 days of surgery, the percentage wound closure was more than 70% in the crossed group, 62% in the aligned 56% in random groups and 40% in control group as observed from Fig. 5.4. The gene expression in fibroblast Col I, Col III, α-SMA, TGF-β1, Integrin α2 and Integrin β1 on cross-aligned fibers were up-regulated by 0.5, 1.0, 1.5, 1.5, 2.0, 2.0 folds respectively (Sun et al. 2018). The delivery of active growth factors can be advantageous to boost healing of the wound (Tanha et al. 2017). Chitosan nanoparticles loaded with Human Granulocyte Colony-Stimulating Factor (G-CSF) were integrated into nanofibers of PCL. This scaffold was coated with collagen type I [PCL/NP (G-CSF)] and was used to cover the full thickness wound surgically created on rats and the percentage wound closure was measured after debridement. After 7 days of creating a wound, 52 ± 0.5% wound closure was observed by PCL/NP (G-CSF) scaffold, while 44 ± 0.4% by PCL/NP nanofibrous scaffolds and 40 ± 0.4% by positive control (Tanha et al. 2017). Eudragit (commercial polymer used to tune the desired type of drug release) nanofibers carrying gentamicin (GS) and immobilized recombinant human

epidermal growth factor (rhEGF) were evaluated on mice. It showed 85.69 ± 2.61% wound closure on the fourth day after surgery. The wound healing efficiency of this scaffold was higher compared to the group treated with the scaffolds without rhEGF and with pure GS ointment (Dwivedi et al. 2018). *In vivo* efficacy of nanofibers made up of PLGA releasing epinephrine, lidocaine, and collagen was examined on palatal oral wound surgically created on Rabbit for pain relief and hemostasis. 67% initial hemostasis (1 min after the surgery) was observed in the test group, but only 17% in control. Furthermore, 100% was observed 10 mins after surgery in the test group. On the other hand, the control group showed 83% hemostasis (Lee et al. 2017). Thus, from the current studies, it can be observed that composite nanofibers and functionalized nanofibers are more advantageous than single polymer nanofibers as scaffolds used to prepare devices enhancing wound healing. Table 5.2 contains a list of some recent *in vivo* studies done using scaffolds made with electrospun nanofibers for their wound healing efficacy.

Nanofibrous scaffold based skin regenerative medicine has many benefits. The nanofibers can be functionalized before or after fabrication. Before fabrication two polymers can be blended i.e., natural or synthetic and can be fabricated in the form of nanofibers. The nanofibers can be coated with desired molecules post fabrication and surface modified. Co-axial nanofibers provide properties of different polymers fabricated together in the form of core-shell nanofibers.

Thus, nanofibers stand as promising regenerative medicine for the skin as it provides mechanical strength which aid to heal full thickness wound. It has a favorable topology and porous structure. Drug-loaded nanofibers facilitate controlled release of the drug. Also, the nanofiber-based scaffold can be used as implant material as well as dressing material according to the need, functional target, type and location of the wound. Further, the diameter of nanofibers can be regulated by changing fabrication parameters such that it matches collagen diameter and helping for the faster wound healing. Thus, many aspects of nanofibers make it suitable to be used for tissue engineering and with time it has been emerging as a good regenerative medicine for the skin.

5.4 Bone Tissue Engineering

5.4.1 Human Bone: Anatomy and Physiology

Bone is a living, mineralized and rigid connective tissue granting strength and skeletal frame to the vertebrates. It is composed of osteoblasts, osteoclasts, osteocytes, and bone lining cells (Florencio-Silva et al. 2015). Providing support, shielding soft tissues from damage, movement, storing calcium and phosphate, and contains bone marrow are the vital roles of bone (Robling et al. 2006). Understanding the anatomy and physiology of the bone is essential for understanding bone metastasis mechanisms and for the development of therapeutic interventions. The generally bones are classified as short bones, long bones, irregular bones, and flat bones. Long bones have the phalanges, fibulae, femurs, tibiae, metatarsals,

Table 5.2 Electrospun nanofibers used for enhancing wound healing: *In vivo* study

Composition	Wound type	Animal model used	Mechanism of action	References
Arginine modified chitosan nanofibers	Full-thickness wound	Rat	Collagen deposition, re-epithelialization, and angiogenesis	(Hoseinpour Najar et al. 2017)
PCL nanofiber with nanosilver	Full-thickness wound	Mice	Re-epithelialization via enhancing keratinocyte proliferation and its antibacterial property in infectious wounds	(Liu et al. 2017)
Multi-layered epinephrine, lidocaine, collagen eluting PLGA-nanofibers.	Palatal oral wound	Rabbit	Pain relief and hemostasis	(Lee et al. 2017)
Eudragit nanofibers carrying gentamicin and recombinant growth factor	Chronic wound—in diabetic model	Mice	Bacterial infection inhibition and helping in the initial stage of wound healing (cell adhesion and proliferation)	(Dwivedi et al. 2018)
Arginine modified Lignin nanofibers	Full-thickness wound	Rat	Increase re-epithelialization, collagen deposition, angiogenesis, and accelerated wound closure	(Reesi et al. 2018)
Crossed patterned PCL-collagen I nanofibers	Full-thickness wound—in diabetic model	Rat	Acceleration of keratinocytes and fibroblasts migration, resolving inflammation, and promoting angiogenesis	(Sun et al. 2018)
Chitosan nanoparticles loaded with G-CSF and PCL nanofibers composite, coated with collagen type I	Full-thickness wound	Rat	Enhanced collagen deposition, re-epithelization, fibroblast maturation, and minimal inflammation	(Tanha et al. 2017)
DMOG releasing nanofibers	Chronic wounds in diabetic model	Rat	Accelerating angiogenesis, re-epithelialization, and wound closure	(Gao et al. 2018)
Bixin-loaded polycaprolactone	Full-thickness wound	Mice	Promoting tissue remodeling	(Pinzón-García et al. 2017)
Soy protein hydrolysate (SPH) and cellulose acetate (CA) co-spun nanofibers	Full-thickness excisional wound	Mice	Accelerating epidermal thinning, re-epithelialization,	(Ahn et al. 2018)

(continued)

Table 5.2 (continued)

Composition	Wound type	Animal model used	Mechanism of action	References
			reduction in scar formation	
Chrysin loaded PCL–PEG–PCL co-polymer nanofiber	Open excision wound	Rat	Wound healing through anti-oxidant and anti-inflammation	(Mohammadi et al. 2017)
Aloe vera gel coated PLLA nanofibrous scaffold	Full-thickness wound	Mice	Hyper-granulation, epidermis and dermis separation increase in collagen deposition, lowering inflammation	(Jouybar et al. 2017)

rhEGF recombinant human Epidermal Growth Factor, *G-CSF* recombinant human Granulocyte Colony-Stimulating Factor, *PCL* Polycaprolactone, *PLGA* Poly (Lactic-co-Glycolic Acid), *PEG* Polyethylene Glycol, *PLLA* poly-L-lactic acid

metacarpals, ulnae, radii, clavicles and humeri. Short bones comprise the sesamoid bones and patellae, tarsals and carpals. The mandible, skull, scapulae, ribs, and sternum are examples of flat bone. The hyoid, coccyx, sacrum and vertebrae are examples of irregular bone. Diaphysis, metaphysis, and epiphysis are present in long bone. The diaphysis also known as hollow shaft constitutes of mainly dense cortical bone, on the other hand, the epiphysis (existing above the growth plates) and metaphysis (existing below the growth plates) are composed of trabecular bone network enveloped by a thin cover of cortical bone. Cortical bone has endosteal as an inner layer and periosteal as outer surface playing a crucial role in fracture repair and appositional growth. Trabecular bone and cortical bone are normally constructed of collagen fibrils arranged in alternating orientations forming a lamellar pattern. Via thick collagenous fibers, the periosteum is firmly connected to the outlying cortical layer of bone called Sharpeys' fibers. These Sharpey's fiber extends to bone tissue underlying. The endosteum containing blood vessels, osteoblasts, and osteoclasts covers the inner surface of the bone tissue (Clarke 2008). Bone is composed of inorganic salts mainly of phosphate and calcium ions which form Hydroxyapatite and the Extracellular Bone Matrix (EBM) (Boskey et al. 2002). The EBM is largely made up of collagen mainly of type I collagen, and other proteins like osteocalcin, osteonectin, osteopontin, bone sialoprotein II, fibronectin, and growth factors like bone morphogenetic proteins (BMPs) (Aszódi et al. 2000). The noncollagenous proteins along with collagen form a frame for hydroxyapatite deposition. This kind of interaction results in the typical resistance and stiffness of the bone tissue (Datta et al. 2008).

5.4.2 Fracture Repair Process

Bone is a very dynamic organ and its remodeling is a highly complex process which includes the substitution of old bone by the new bone. This process includes synchronized functioning of the basic multicellular unit (BMU) which is the provisional anatomical complex of osteoclasts, osteoblasts, osteocytes, and the cells lining the bone perform a series of three phases: (1) Resorption of bone by osteoclasts (2) Resorption to new bone formation transition phase (3) Osteoblasts forming the new bone (Matsuo and Irie 2008). Fracture repair follows either of the two processes; direct or primary fracture repair and secondary fracture repair.

Primary fracture repair follows when the distal part of the fractured bone lack relative displacement and are rigidly fixed, leading to no or little inflammatory response. This includes the deposition of bone tissue: osteoblasts and mesenchymal progenitor cells rush to the injury site and get activated and they unify the tissue by depositing a bone matrix (Ai-Aql et al. 2008; Loi et al. 2016). Secondary fracture repair is the common fracture healing observed in the clinic in which bone repair takes place via cartilaginous intermediate and in various phases (Inflammatory Phase, Cartilaginous Callus Formation, Bony Callus Formation, and Remodeling Phase). Immune cells have a major part in the secondary bone repair process (Baht et al. 2018). The phases of secondary bone repair are discussed in detail below.

Inflammatory Phase

In the case of Long-bone fracture, there is a local damage in the vasculature and the soft tissue. Thus, leading to a hematoma formation which acts as the blueprint for callus construction. Followed by the recruitment, activation, and invasion of immune cells including neutrophils, platelets, and macrophages to the hematoma which secretes the cytokines and the growth factors to recruit mesenchymal cells. The hematoma is then reorganized along with fibrin thrombus deposition (Kolar et al. 2010; Marsell and Einhorn 2011; Ozaki et al. 2000). Further, capillaries invade the thrombus and the fibrin clot is replaced by the granulation tissue. Dead cells and debris are cleaned up by the macrophages and neutrophils (Thomas and Puleo 2011). The recruitment of mesenchymal progenitor cells originates from the periosteum, bone marrow, and systemic circulation (Colnot et al. 2006) that is aided by the factors released from the immune cells. In turn, these cells acquire an immunosuppressive character which assists to resolve the inflammation at the site and makes a path for the succeeding phase of healing (Jiang et al. 2005).

Cartilaginous Callus Formation

The reduced mechanical integrity at the fracture loci encourages the chondrogenic differentiation of the mesenchymal progenitor cells. After that, the granulation tissue is substituted by a semi-rigid callus which has fibrous and cartilaginous characteristic providing mechanical support (Marsell and Einhorn 2011; Schindeler et al. 2008). This callus is avascular to a large extent; but as repair mechanism progresses, the endothelial cells invade the callus, promoting angiogenesis (Carano and Filvaroff 2003). This results in the terminal differentiation of chondrocytes which initiate the

generation of the mineralized cartilaginous matrix (Dimitriou et al. 2005; Schindeler et al. 2008). These chondrocytes either undergo apoptosis or dedifferentiate into osteogenic cells (Yang et al. 2014).

Bony Callus Formation
The bone marrow, periosteum, vasculature, and neighboring tissue are the sources for Osteoprogenitor cells. They commence osteogenesis to deposit bone upon the calcified cartilaginous callus (Schindeler et al. 2008). Simultaneously, cartilaginous callus is substituted by the bony callus on the activation of the osteoclasts at the site. It constitutes of woven bone which has more stability than the cartilaginous callus (McKibbin 1978).

Remodeling Phase
It is the last stage of fracture repair in which the laminar bone replaces the woven bone present within the callus. It consists of a highly organized matrix of collagen fibers, restoring the native function and structure of the bone (Marsell and Einhorn 2011). The act of bone formation and bone resorption is conducted by osteoblasts and osteoclast, respectively (Schindeler et al. 2008).

5.4.3 Types of Bone Fracture

The bone gets a break or splits if more pressure is put on it than it can withstand. A break in the bone of any size is called a fracture. The bone fracture is classified into various types based on the broken bone as shown in the Fig. 5.5. Compound/open in which fracture breaks the skin and Simple in which fracture does not break the skin. Comminuted in which the bone gets fractured into small fragments and it is the most difficult fracture to heal. In Greenstick type, on one side the bone is fractured and it tilts on the other side. The fracture in which end of the bone gets forcibly inside the

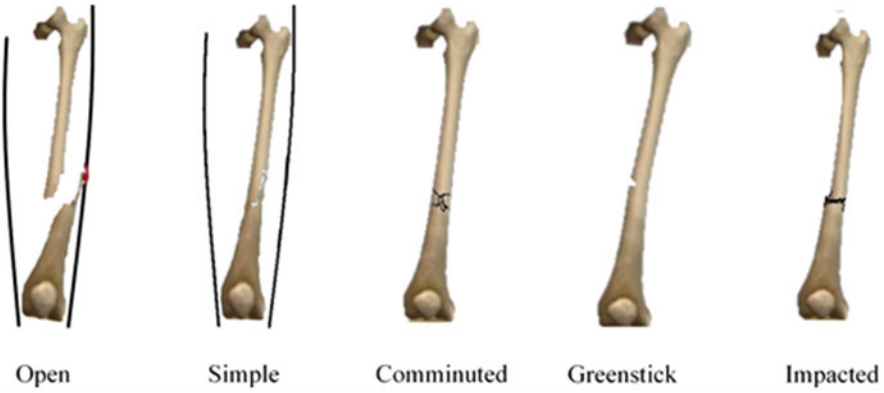

Fig. 5.5 Types of bone fracture according to the broken bone

other bone is called Impacted fracture. A distal fibula fracture is called Pott's fracture and a fracture at the extreme end of the radius is called Colles' fracture. Also, a series of minute fissures formed in the bone, without imparting any damage to any other tissues is called Stress fracture (Tortora and Derrickson 2008). The critical sized defects are defined as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal" (Schmitz and Hollinger 1986) or the one which shows <10% regeneration of the defected bone during the lifespan of the animal (Gugala and Gogolewski 1999).

5.4.4 Conventional Methods Used to Repair a Bone Fracture

The bone can repair and regenerate itself (Nandi et al. 2010). However massive and large bone defects are unable to heal completely. Many factors can influence bone healing negatively such as infection of the surrounding tissues or the bone, insufficient blood supply, resulting in delayed unions or non-unions (Bigham et al. 2008; Scaglione et al. 2014). Bone is the second most transplanted tissue after blood (Nandi et al. 2010). A material implanted which helps in bone healing in combination with some other material(s) or independently is known as a Bone graft (Elsalanty and Genecov 2009), through osteoconduction, osteoinduction, osteogenesis and osteointegration (Albrektsson and Johansson 2001). Osteogenesis is the property of osteoblasts to induce osteoprogenitor cell differentiation to produce new bone either existing in the recipient's bone or made available with the help of the graft material. Osteoconduction is a feature of the graft which can aid new bone formation by providing mechanical support and blood vessel development from the borders of the fracture (Albrektsson and Johansson 2001; Di Martino et al. 2011; Keating and McQueen 2001; Parikh 2002). Osteoinductive graft induces multipotent mesenchymal stem cells (MSCs) of the nearby host tissues to differentiate into osteoprogenitor cells and then production of osteoblasts. This process is facilitated by the growth factors including transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF). BMP-2 and BMP-7 (bone morphogenetic proteins—BMPs). Osteointegration is the capacity of the graft to bind to the neighboring bone without an intervening layer of fibrous tissue and getting incorporated at the host site. Bone grafts may be cortical- for strength and structural support, cancellous- for osteogenesis, or corticocancellous- having properties of both kind (Brydone et al. 2010). Conventionally used bone grafts are categorized into autografts, allografts, and xenografts. The Autograft is harvested by the surgeon from some other location of the patient's body, usually from the iliac crest, and implanted to the defected bone loci of the same person (Rose and Oreffo 2002). Autografts have strong osteogenic characteristics for bone remodeling and thus considered as 'gold standard' for small bone defects (Athanasiou et al. 2009). Further, Xenografts is acquired from an animal model and Allografts are the bone grafts obtained from the human donor.

5.4.5 Demand for Engineered Bone Substitutes

Tissue engineering employs relevant scaffolds to introduce cells, more recently the stem cells and appropriate growth factors (Moshiri and Oryan 2012) aiming to improve graft properties and overcome the limitations of traditionally used grafts. Among various types of conventional bone grafts, autografts have all the four characteristics osteogenic, osteoconductive, osteoinductive, osteointegration. Whereas, Allografts and Xenografts exhibit only osteoconductive and osteoinductive property but lack the osteogenic and osteointegration properties of Autografts (Dimitriou et al. 2011; Keskin et al. 2007). Whereas, Autograft has disadvantages like donor site morbidity, pain, injuries to major vessels while harvesting the Autograft (Ehrler and Vaccaro 2000). Moreover, it requires two surgeries, thus additional infection risk, two scars, and more pain. While Allografts have major limitations like high cost, the risk of diseases transmission, low incorporating properties with the host healing tissues resulting in rejection. Similarly, Xenografts have certain shortcomings like chances of rejection is high, and carries the risks of transmission of zoonotic diseases to the host (Moshiri and Oryan 2012; Oryan et al. 2013). Whereas, engineered bone graft substitute made up of biomaterials do not require a second surgery site and are immunogenically safe. Tissue engineered bone grafts are a potentially powerful strategy as bone regenerative medicine. However, to translate research findings into a clinical application requires *in vivo* models to explore the bone regenerative properties of the tissue engineered bone grafts.

5.4.6 Bone Fracture Repair Study Models

Various factors need to be kept in mind while selecting an animal species as a model system include costs for care and acquisition, availability, ease of housing, tolerance to captivity and ethically permitted (Pearce et al. 2007). For bone repair study, the preferred animal model should have significant pathophysiological and physiological analogies to humans. For example, species phylogeny, age, defect size, anatomic location, the presence of periosteum, bone structure and vascularization, mechanical stresses on the limb, systemic conditions, adjacent soft tissue, stiffness, nutrition which may govern critical size bone defect repair (Reichert et al. 2009). Over the last decades, the dog has been considered as a suitable animal model by several publications related to human bone defect conditions (Jung et al. 2015; Khojasteh et al. 2017; Martini et al. 2001; Zhu et al. 2016). In the context of bone density, weight, and its material constituents, dogs were the closest to humans. But there is a clear description of differences in bone microstructure and remodeling between dogs compared to humans (Aerssens et al. 1998; Gong et al. 1964). Adult human bone has a secondary osteonal structure. This structure has osteons greater than 100 μm and consists of blood vessels and a boundary between adjacent lamellae formed by the cement line. Whereas, canine bone have a combinational microstructure mainly the secondary osteonal bone, but in the areas adjacent to the periosteum and endosteum

there occurs plexiform bone (Wang et al. 1998). Canine trabecular bone can withstand higher compressive strains than human bone before failing (Kuhn et al. 1989). The utilization of dogs as *in vivo* model has remarkably reduced due to the ethical problems (O'loughlin et al. 2008). Mature sheep and goats are also used to study human implants as they possess long bone and their body mass is comparable to adult humans and their mechanical loading environment has been well understood (Newman et al. 1995; Taylor et al. 2006). Mineral composition (Ravaglioli et al. 1996), metabolic rate and bone remodeling are also alike to humans (Anderson et al. 1999). However, there are some differences revealed in bone structure between Sheep and humans by the histology. Sheep have primary bone structure, the osteons are of less than 100 μm diameter. Cement line is absent and contains not less than two central blood vessels (De Kleer 2006). In comparison with humans having the mainly secondary bone structure (Eitel et al. 1981). Femur trabecular bone density is 1.5–2 fold greater in Sheep than that of humans (Eitel et al. 1981; Liebschner 2004).

Moreover, Guinea pig, Rat, and Rabbit are also utilized to investigate the bone graft substitutes, the effect of the growth factors and macroscopic/microscopic changes after treatment. The Rabbit is one such small animal having similar anatomical sites to humans such as long bone, lumbar spinal and adequate iliac crest bone which can be used as an autogenous graft for the study purpose (Oikarinen 1982; Rauch et al. 2000). Moreover, the use of Rats has been restricted to few anatomical locations like femur (Guizzardi et al. 1992; Yasko et al. 1992). Usually, tests are limited to compression of vertebral bodies and bending of long bones in rats. Determination of mechanical characteristics of trabecular bone in rats is difficult, but not impossible. However, human pathophysiology is not mimicked perfectly by any of this animals because of variations in hormone profile, physiology of reproductive system, biological and mechanical bone property. As a matter of fact, more information generated by conducting studies using small *in vivo* model may be helpful than the less number of data obtained from a large *in vivo* model (Liebschner 2004).

5.4.7 Scaffolds for Bone Tissue Engineering

Tissue engineering approach used to create Regenerative medicine aims to repair or substitute malfunctioned/damaged tissues or organs (Gurtner et al. 2007). For tissue engineering, scaffolds are the most crucial part. The inner architecture of the scaffold, cellular penetration, extracellular matrix production, and neovascularization facilitation are very important. A 200–350 μM pore size can be considered the most favorable for inducing osteoconduction and bone development (Whang et al. 1999). Over porous scaffolds and hydrogels, fibrous scaffolds are preferable as they mimic the native ECM fibrous structure, aiding to suitable morphologies (Alvarez-Barreto et al. 2007). Nanofibers possess high isotropic and porous structure and possesses homogenous fiber diameter. Also, they show similarity to the collagen fibril network each of about 50–500 nm diameter. In addition, nanofibrous scaffold assist cell adhesion and proliferation, as the fibers have a

diameter less than that of the cells, they can organize and adhere well around the fibers (Nair et al. 2004). Furthermore, nanofibrous scaffolds can have varied mechanical properties, depending upon fiber diameter, its composition, and orientation (Vatankhah et al. 2014). Also, their controlled patterned structures can support cell for adhesion, migration, proliferation, and differentiation (R. Ng et al. 2012; Pina et al. 2015). The scaffolds made up of fibers can be classified into two main groups i.e., natural or organic and synthetic materials. While the issue of biocompatibility of synthetic material is solved by combining both synthetic and natural polymers well known as the composite or hybrid materials. This kind of material has better scaffold properties than the one made up of natural or artificial alone (Moshiri and Oryan 2012).

Bone regenerative medicine uses the scaffolds which provides the three-dimensional structure like by the ECM in the native state, thus supporting the bone regeneration. Later these scaffolds degrade after the new bone formation. 3D scaffolds fabricated using natural polymers have characteristics like as biodegradability and biocompatibility, which is suitable when used as regenerative medicine. Charge, porosity, mechanical strength, and bioactivity of the fibers can be directed according to the requirement by varying polymer concentration, reaction conditions, by functionalizing or by adding peptides, proteins, cells, and chemicals (Lee and Yuk 2007; Lee and Shin 2007). Natural polymers mostly explored for bone tissue engineering are collagen, hyaluronic acid, gelatin, silk, alginate, chitosan, peptides (Vagaská et al. 2010) elastin, cellulose (Oryan et al. 2014).

Synthetic polymers are artificial polymers. The no or less biodegradability of the synthetic material is its major limitation which leads to its reduced usage translationally. Biodegradable property can be conferred to these materials by controlling the conditions during their production (Dhandayuthapani et al. 2011). Materials like coral derivatives, bioactive glasses are also used. Polyglycolic acid (PGA), polylactic acid (PLA), poly(ε-caprolactone), and hydroxyapatite (HA) and tricalcium phosphates (TCP) derived from calcium-phosphate are also included in synthetic materials (Polo-Corrales et al. 2014).

At present, composite grafts include growth factors (BMPs) or bone-marrow aspirates, such as MSCs or mature cells seeded with bone synthetic or bioabsorbable biocompatible scaffolds. Thus, emerging as an alternative to autologous graft for bone (Jones and Yang 2005). There are reports about the use of polymer coated metallic implants. Scaffolds composed from Ti-alloy, Titanium dioxide, or Titanium along with other polymer coating has been broadly studied. For instance, an implantable scaffold was fabricated coating titanium with polylactide, hydroxyapatite, and $CaCO_3$. On further investigation, it showed mechanical stability, partial biodegradability and biocompatibility (Lagoa et al. 2008). Another example of a composite polymer is bioactive glass ($CaO–P_2O_5–SiO_2$) with collagen which showed high bone defect healing capability (Xu et al. 2011).

5.4.7.1 Nanofibrous Scaffolds for Bone Tissue Engineering: *In vivo* Studies

In vivo investigation is very crucial for the development of medical devices as it provides proof-of-concept, function validation and information for further clinical trials. Thus, studies are performed on large or small animals for the development of regenerative medicines. For that bone defects are created in the *in vivo* models. Bone can get fractured at the strain of about 0.3 and 1 (Currey 2002). The fabricated devices are implanted on the defect site to validate their regeneration potential. Some recent studies involving electrospun nanofibers for bone regeneration has been discussed below.

Silk fibroin (SF), a natural structural protein has been researched extensively for controlled drug delivery (Kundu et al. 2013) and tissue engineering (Yucel et al. 2014). Silk scaffolds were reported for their cell adhesivity, resistance to enzymatic degradation, mechanical strength, and permeability (Kim et al. 2014). Icariin is the key bioactive component in the total flavonoid extraction from the Chinese traditional herb *Epimedium brevicornum* Maxim. For bone tissue engineering, Icariin (ICA) encourages the proliferation and differentiation of osteoblasts (B. Liu et al. 2011) and is considered as an osteogenic inducer. Nanofibrous ICA-SF/PLCL membrane was formed by electrospinning it co-axially and evaluated for Cranium defect in Rat. Twelve weeks post-implantation, the test group showed new bone formation covering most of the defect area, with a volume of about 15.95 ± 3.58 mm^3. Whereas, control group without any implant showed approximately 1 mm^3 and negative control group which was treated with nanofibrous SF/PLCL membrane showed around 8 mm^3 defect coverage. The percentage healing density was approximately $14.02 \pm 0.93\%$ in the test group, in the negative control group around 8% and about 1% in the control group (Yin et al. 2017).

In another co-axial nanofiber based study, SF–PCL shell and PRP–PVA in the core were evaluated in Mice cranial defect. The platelet-rich plasma (PRP) contains a high amount of growth factors advantageous for healing wound and tissue regeneration. Among them, VEGF, TGF-β, insulin-like growth factor (IGF), PDGF enhance bone regeneration (Roussy et al. 2007). Eight weeks after surgery, the cranial bone defect was analyzed by μCT scanning. In the PRP-NFS treated group, the new bone tissue almost filled most of the cranial defect; the bony callus was reconstructed compared to the group treated with nanofibers without PRP and the control groups which were not treated with any nanofibers which remained unfilled. This study showed the osteogenic potential of PRP-derived growth factors promoting mineralization and osteogenic differentiation of BMSCs facilitated by the controlled release of PRP incorporated in the co-axial nanofibers (Chen et al. 2018). These studies clearly demonstrated the role of natural polymers in controlled release of biomolecules as well as ECM mimicking potential for bone regeneration.

BMPs delivery for bone graft substitutes through biomaterials is a fostering approach for bone defect repair. BMP-2 and BMP-7 are the two FDA approved growth factors, widely been used in orthopedic applications clinically due to their potential to induce bone formation and osteogenesis (Khosla et al. 2008; Schroeder and Mosheiff 2011; Zheng et al. 2010). Nanofibers composed of PEO and BMP-2 in

the inner core and c-6A PEG−PCL nanogel (NG) with −S−S−bond as outer shell were fabricated by coaxial spinning. The BMP-2 release can be regulated by the redox-sensitive outer shell of the nanofibers as the shell permeability changes in response to the change in the GSH (glutathione) concentration. This nanofibrous scaffold was examined for the *in vivo* critical sized mandible defect repair in Rat. The percentage bone repair after 12 weeks of surgery of the control group, groups treated with PCL nanofibers, BMP-2, PCL/BMP-2, PCL/NG/BMP-2 nanofibers were approximately 9%, 12%, 19%, 35%, and 79% respectively. Thus, this study demonstrated the programmed delivery of BMP-2 achieved using a redox-sensitive nanofiber (Gong et al. 2018).

Hydroxyapatite (HA) is widely utilized for scaffold modification used in bone tissue engineering. This is because it can mimic the biphasic architecture of natural bone (Venkatesan and Kim 2014). The major advantage of PCL nanofibers is their unique surface area to volume ratio. Thus, it can be employed as carriers for local delivery of biomolecules like hydroxyapatite (Kanungo et al. 2013). A 3D scaffold was generated of electrospun PCL nanofibers coated with HA and used in combination with Phenamil (BMP-2 signaling activator) and BMP-2. The histological observation after 4 weeks of surgery revealed that the test group showed a 43% new bone area, whereas the control group showed about 18% new bone area. PCL/HA-3D scaffolds along with phenamil and BMP-2 synergistically enhanced osteogenic differentiation (Miszuk et al. 2018). Another study was carried out using electrospun PBAT [poly (butylene-adipate-coterephthalate)] and nHAp nanofibers. PBAT has low crystallinity, good biodegradability, and mechanical characteristic. Moreover, synthetic Nano-hydroxyapatite (nHAp) is bioactive and osteoconductive. *In vivo* study using this scaffold in Rat tibial defect showed that the bone volume increase was 0.7 mm^3, 1.2 mm^3 and 1.6 mm^3 in control group, group treated with only PBAT fibers and group treated with PBAT fibers loaded with 3% nHAp respectively. Also, 3D reconstructed micro-CT after 6 weeks of scaffold implantation showed better bone repair in PBAT/3% nHAp treated group than in only PBAT treated group and control group (Santana-Melo et al. 2017). In another mineral based study, CMCS (Carboxymethyl chitosan) along with PEO were electrospun. CMCS is a water-soluble derivative of chitosan. Chitosan is made up of GlcN, which is also a major component of the glycosaminoglycans (GAG) present in the ECM. Chitosan has a positive charge due to its amino group, thus it can bind the cell membrane (Sivashankari and Prabaharan 2016). Moreover, CMCS has better bioactivity and biodegradability than normal chitosan. Thus, CMCS electrospun nanofibers were explored in this study. These nanofibers had mineralization property, thus aiding to form CMCS-HA nanofibers. The osteogenic effect of this nanofibers was tested on a critical-size defected calvarial bone in Rats as *in vivo* model. The expression level of osteogenic genes Runx2 and ALP were approximately 1.6 and 4.3 folds after 7 days, and 5.1 and 10 folds after 14 days respectively, on CMCS-HA nanofibrous membranes compared to that on CMCS samples. The new bone feeling at Rat calvarial defects at 4 weeks and 12 weeks post surgery was analyzed using Micro-CT scanning as observed in Fig. 5.6. In the CMCS-HA nanofiber treated group, the entire defect was almost filled by newly formed bone after 12 weeks compared to

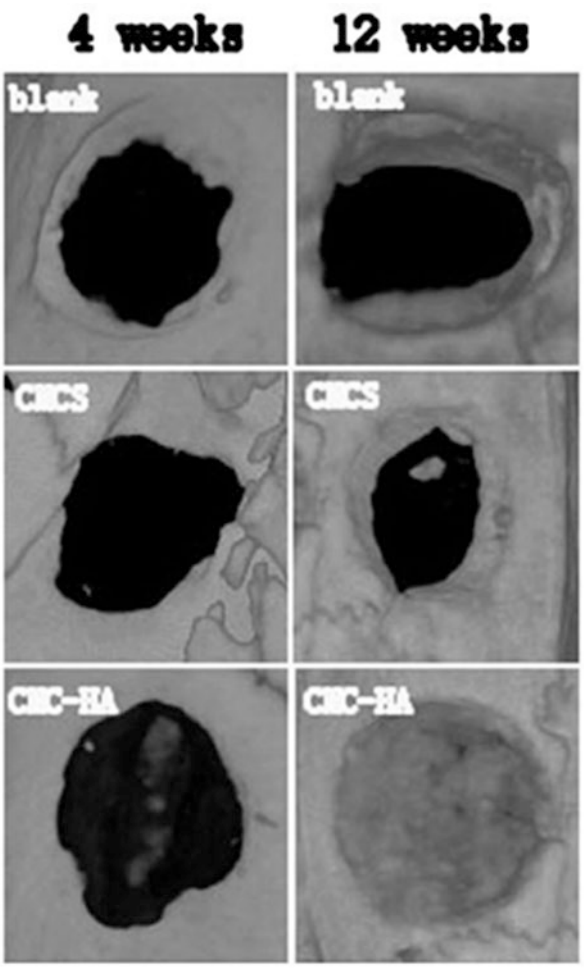

Fig. 5.6 Study of Rat calvarial defects repair using Micro-CT in blank, CMCS, and CMCS-HA treated group at 4 weeks and 12 weeks post surgery. Reprinted with the permission from Ref. (Zhao et al. 2018) copyright © Elsevier

only CMCS treated group and blank group i.e., without implanting any material. Thus, depicting osteogenesis enhanced by CMCS-HA composite nanofibers (Zhao et al. 2018). It can be concluded that HA can change the physicochemical properties of graft materials resulting in enhanced cell adhesion, proliferation, and migration. Scaffolds incorporating HA can serve as a support system for enhanced bone regeneration.

The mechanical instability and uncontrolled swelling in aqueous environments are the limitations of chitosan-based nanofiber membranes in guided bone regeneration. Therefore, a study was conducted in which modification of chitosan nanofibers with surface butyrylation lead to enhanced stability by providing hydrophobicity as its site of action will be an aqueous environment. The CSNF (Chitosan nanofiber) was surface butyrylated post fabrication (BCSNF) and studied for healing calvarial critical size defect in Rats for guided bone regeneration (GBR). The

histomorphometry showed percentage new bone volume/defect volume as 4.0 ± 5.3% in control group treated with commercial collagen membrane and 10.2 ± 12.1% in the test group treated with BCSNF post 3 weeks of surgery and 24.8 ± 6.5% and 37.2 ± 22.7% respectively, after 12 weeks of surgery. BCSNF enhanced bone regeneration and showed good barrier function in the bone regeneration process (Chaoxi et al. 2018).

Titanium (Ti) implants have been extensively used in orthodontic and orthopedics surgeries (Wu et al. 2013). Ti implant surface is inert but lacks native tissue-tissue interfaces. This is because there is no osteogenesis regulating and enhancing biological entity at tissue-Ti implant-interface. Apart from employing them as a stand-alone regenerating device, nanofiber coating on metallic implants was studied for augmenting their biocompatibility and tissue integration. In a study, Ti wire surface coated with Collagen and aligned PCL nanofibrous matrix were implanted in Rabbit femur. From the *in vivo* studies, it was found that the mean cortical bone volume difference between NFM coated and control was 12.31. Thus, the interface shear strength of CG-PCL NFM-coated Ti/bone samples was significantly higher than control Ti/bone samples. The obtained result was due to the relatively enhanced growth of the connective tissue at the Ti implant and tissue interface. Thus, the use of electrospun nanofiber matrix leads to improvement in the biomechanical performance of titanium (Ti) and its biocompatibility (Khandaker et al. 2018). Table 5.3 describes summarized information about some recent *in vivo* studies done using scaffolds based on electrospun nanofiber for bone tissue regeneration.

Bone regenerative medicine based on nanofibers has many advantages. The nanofibers can be functionalized before or after fabrication. The targeted and controlled release of bioactive content from natural extracts can be facilitated by electrospinning the plant extracts and enhancing its effectiveness by converting it into nanoscale. The co-axial fibers can provide properties of two or polymers aiding to the synergistic effects of many osteogenic components to the target site. Also, providing the desired characteristics to the fabricated device. Electrospun nanofibers have porosity and surface topography similar to native ECM and thus, it enhances cytocompatibility. In the case of bone regeneration applications, the nanofibers facilitate osteoblast cell adhesion, proliferation, protein adsorption, and mineralization. Also, as it has nanoscale and high surface area to volume ratio, appropriate and controlled amount of the growth factor, BMSCs and another biomolecule can be delivered at the bone defect site which can be cost effective when compared to non – targeted delivery. The bone implants can be coated with nanofibers in the form of a simple coat, in aligned pattern and with post coating modification.

These assists the metallic implants for better tissue integration by increasing mechanical strength, surface biocompatibility. Due to so many advantages, nanofibers can be considered a boon in the advancing field of bone regenerative medicines. Through *in vivo* studies, the biocompatibility, biofunctionality, and bioinertness of the newly synthesized biomaterial can be assessed. The *in vitro* results validated further by *in vivo* assessment provides proof of concept of the proposed biomaterial is to be utilized as a regenerative medicine.

Table 5.3 Electrospun nanofibers used for enhancing Bone tissue regeneration: *In vivo* studies

Composition	Type of bone defect	Animal model used	Mechanism of action	References
PCL nanofibrous scaffolds grafted by non-mulberry silk fibroin along with HA and covalently coupled BMP-2 and TGF-β	Distal metaphysis region of femur	Rabbit	Osteoblast proliferation and differentiation, mineralization	(Bhattacharjee et al. 2017)
SF/PCL/PVA nanofibers consisting of PRP-derived growth factors	Cranial defect	Mice	Migration, proliferation, and osteogenesis of BMSCs *in vivo*	(Cheng et al. 2018)
PCL, gelatin, nanosized HA, dexamethasone, ascorbic acid and beta-glycerophosphate nanofibrous coating on titanium implant	Implants at proximal diaphysis and in the central diaphysis	Rabbit	Enhancing osteointegration, osteoconduction, and osteoinduction	(Das et al. 2018)
c-6A PEG−PCL nanogel with −S−S−bond (outer shell) PEO BMP-2 (inner core)	Mandibular defect	Rat	Osteogenesis by osteogenic differentiation of BMSCs by programmed delivery of BMP-2	(Gong et al. 2018)
Ti surface coated with collagen and PCL nanofibrous matrix	Implant in femur	Rabbit	Increasing cytocompatibility of Ti and facilitating osteointegration of the implant	(Khandaker et al. 2018)
Electrospun PCL nanofibers generated 3D scaffolds coated with HA and used in combination with Phenamil	Ectopic bone formation	Mice	New bone formation by promoting BMP2 signaling pathway and osteogenic differentiation	(Miszuk et al. 2018)
PBAT [poly(butylene-adipate-coterephthalate)] with synthetic Nano hydroxyapatite (nHAp)	Tibial defect	Rat	Bone regeneration by osteoinduction and osteoconduction	(Santana-Melo et al. 2017)
Surface butyrylated chitosan nanofiber (BCSNF)	Calvarial critical size defect	Rat	Adhesion and proliferation of fibroblasts and bone regeneration	(Chaoxi et al. 2018)

(continued)

Table 5.3 (continued)

Composition	Type of bone defect	Animal model used	Mechanism of action	References
ICA-SF/PLCL nanofibrous membrane	Cranium defect	Rat	Inducing bone regeneration by activating the proliferation and differentiation of osteoblast leading to osteogenesis and regulating osteoclast	(Yin et al. 2017)
CMCS-HA composite nanofibers	Critical-size calvarial bone defect	Rat	Promoting osteoblastic differentiation of progenitor cells leading to osteogenesis	(Zhao et al. 2018)

PLCL poly (L-lactide-co-ε-caprolactone), *ICA* Icariin, *CMCS* Carboxymethyl chitosan, *HA* Hydroxyapatite, *SF* Silk fibroin, *PRP* Platelet-rich plasma, *Ti* Titanium

5.5 Clinical Applications

To provide a practical update on the application of electrospun nanofiber based potential regenerative medicine for skin and bone injuries, a number of evidence obtained from human randomized and controlled clinical trials are critical. This provides information about their translational potential, challenges, prospects, and limitations. Recently one such electrospun nanofiber based wound dressing is in a clinical trial. The SpinCare™ system by Nicast Ltd. This handheld device produces on the spot *in situ* electrospun nanofibrous dressing for treating the external burns and donor site wounds by its 1 min activation from a distance of 20 cm. It offers optimal wound coverage, no touch treatment to patient leading to a minimal chance of bacterial infection, minimal dressing associated pain, quick and effective treatment and due to its transparency regular healing process can be easily monitored. According to one of the case reports of the clinical trials using SpinCare™ dressing, a patient suffering from a second degree burns 10% total body surface area was healed on day 7 after applying SpinCare™ and dressing was peeled off easily (Dubson 2017). Another such clinical trial has been completed by the medical device EktoTherix™. It is a tissue repair scaffold developed by Neotherix Limited by electrospinning technique aimed for the treatment of dermatologic wounds created while surgically removing non-melanoma skin cancers. This device is implanted into the surgical wound which helps the patients' native cells to repair and enhance the wound healing. It is biocompatible and biodegradable, thus no need for device removal post healing. Thus, such clinical trials of the electrospun based medical device have been demonstrating the vast potential of electrospun nanofiber based dressing to be translationally used for the wound healing. Similarly, looking at the

results derived from *in vivo* studies using an electrospun nanofiber-based scaffold, clinical trials related to bone and other tissue injury are expected in the nearby future.

5.6 Conclusion and Future Perspectives

Regenerative medicine has offered numerous opportunities via tissue engineering. Although it is hard to mimic the native architecture of tissues like skin and bone in a scaffold material. But recent science and technological advancements in the field of biomaterial and biomimetics exhibit a potential to achieve scaffolds with biological functions. The electrospinning technique has proven itself as a suitable option for the fabrication of scaffolds offering a wide range of required properties. The appropriate selection of polymer and synthesis parameters govern their mechanical properties, geometry, pore size and distribution, fiber diameter, scaffolds with fiber deposition in varied orientation, their capability for biomolecules release rate, and degradation. The electrospun nanofiber based medical device can be obtained and used in different forms like film, mesh, gel, the coating on another substrate (controllable coating layers), and can be transparent or opaque. This nanofiber-based scaffold can be used as a guided system for drug delivery, nanoparticles, bioactive molecules, growth factors, and the stem cells. These scaffolds have characteristics mimicking the ECM. As described in the present chapter, the electrospun nanofibers based on different scaffolds natural, synthetic, and composite have properties like biocompatibility and are biodegradable. A combined approach using cells, growth factors, other biomolecules along with biocompatible nanostructured scaffolds, would be the key to success. Numbers of pre-clinical *in vivo* studies done on large and small animals have provided convincing evidences about the hidden possibilities of electrospun nanofibers based medical devices effective as a regenerative medicine. They can confer properties to the nearby environment of the site of device implant and induce re-epithelialization, angiogenesis, collagen deposition, antibacterial property, suitable topography for cell adhesion and proliferation, resolving inflammation in case of skin tissue regeneration. Similarly, electrospun nanofiber based devices are being now extensively studied for bone tissue engineering. Such devices directly implanted or metallic implant coated with electrospun nanofibers placed at the injury site induce bone tissue regeneration through various mechanisms like osteogenic differentiation of BMSCs, osteointegration, osteoinduction, and osteoconduction. For the proof of concept of the electrospun nanofibers based devices as a budding regenerative medicine, extensive clinical trials need to be done of the devices functioning well in the pre-clinical studies. There are very few electrospun nanofiber based devices that have successfully entered to the clinical trial level. Considering the outcomes derived from the related *in vitro*, *in vivo*, and clinical studies to date, further thorough research is required for its bench to bedside application. Due to so many advantages, electrospun nanofiber based medical devices can be stated as a boon in the field of tissue regeneration and furthermore extensive research can lead these scaffolds as potentially better regenerative medicine than the treatments available till date. In Future, it is evident that more promising electrospun nanofiber based devices for skin, bone, and other tissue

regeneration will come up and will translationally function. Better management of tissue engineering with the help of electrospun nanofiber based devices can be anticipated during the upcoming few decades due to the fast pace of advances in the field of biomaterial sciences, nanotechnology and regenerative medicine. Thus, reliable assessment of the electrospun nanofiber based biomedical devices is very crucial in order to solve significant problems and it could stand out as an asset for skin, bone, and other tissue regeneration, ensuring the safe application, fast recovery, and preferred translationally used regenerative medicine in future for better treatment.

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Functional Dendritic Coatings for Biomedical Implants

6

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Abstract

Dendrimers are class of highly branched nanostructures, consisting of a central core, a branched dendritic interior, and an exterior surface possessing multiple functional groups. Their well-defined compact size and shape, multivalent nature, and a high degree of molecular uniformity make them preferred choice of candidates for various biomedical and engineering applications. Dendrimers are utilized for biomedical implant coatings, solubility enhancement, drug-delivery systems, chemical sensors, medical diagnostics, catalysts, separation agents, high-performance polymers, building blocks of supermolecules and, etc. The unique property of dendritic macromolecules, in combination with the other characteristics, have shown significant potential as functional coatings for biomedical implants. These have been utilized in the form of (1) composite, (2) soft coating materials, and (3) template for fabricating biomedical implants. The dendrimer incorporated with implant have offered multifunctional roles including drug loading, antibacterial film, passivating layer, biocompatibility, and osseointegration, etc. This chapter discusses the current state-of-art on various applications of dendrimer for design and development of biomedical implants, wherein the main focus is on properties of dendrimer and their functional advantages. The strategies for fabricating the dendritic soft coating on various

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types of biomedical implant substrates and the development of dendritic nanocomposites, for their use as biomedical implants, will also be highlighted.

Keywords

Dendrimer · Implants · Functional coating · Nanocomposites · Hydroxyapatite

6.1 Introduction

Replacement of bone and tooth with metal or ceramic-based implants and/or plates is one of the most successfully adopted surgical procedures worldwide. According to the reports, more than 1.3 million and more than 700,000 dental-implant procedures are performed in Europe and the United State, respectively (Tomisa et al. 2011). As per a report published in 2010, every year approximately 2.5 million artificial hips and 4.7 million knees are implanted in the US (Maradit Kremers et al. 2015). These surgeries can provide a major improvement in the quality of life and function for those with severe musculoskeletal conditions. On an average, these procedures have the success rate of >90% for hip and knee replacement and 90–95% for dental implants (Garg et al. 2017; Raikar et al. 2017). As per the dental implant market report, the global dental implant market is valued at 4590 million US$ in 2018 and will reach 7980 million US$ by the end of 2025, growing at a CAGR of 7.2% during 2019–2025 (Market watch 2019). Even though the demand and success rates are very high for implants based procedures, there are always many risk factors that can lead to implantation failure (Borba et al. 2017). Typically, the implant failure rate lies in the range of 5–10% over the 5-year period, which depends upon the implant type, disease condition of the patient, surgical procedure, and implant site, etc. (Raikar et al. 2017). In many of these cases, replacement surgeries are performed, which are relatively costly and painful for the patients.

To minimize the implant failures, researchers have investigated various avenues of the implants including the materials type, composite material development, surface texture, and functional coatings, which can improve the integrity of the implant and normal functioning of the tissues (Dehghanghadikolaei and Fotovvati 2019). Because of these efforts, the biomedical implant field has witnessed significant growth in the last two decades and a plethora of improvements and strategies have been evolved, wherein few of them have been commercialized as well. In this context, substantial research has been conducted on functional coatings of the implants inducing the desired response with minimum or no complications such as inflammation, infections, or rejection by the tissues or by means of immune response. In addition, the functional coatings have offered the advantages of (1) greater success with higher longevity (2) drug-eluting ability, (3) faster recovery, and (4) minimal unwanted side effects, etc.

The advances and confluence of various disciplines have shifted the research trends to design a variety of functional coatings with naturally active/mimic molecules rather than the whole structures. These molecules include polymers,

dendrimers, aptamers, peptides, fluoride and various other chemicals orbiochemicals (Abou Neel et al. 2016; Lin et al. 2015; Terada et al. 2018). Although many of these coating materials have demonstrated certain types of advantages, dendrimer has received considerable attention as a multifunctional coating material, especially for bone and dental implants. This is primarily due to their distinct structural and dendritic properties along with ease of functionalization and immobilization. Hence, in this chapter, we highlight the impact of dendrimer as functional biomedical implant coatings and the promises that have been successfully accomplished. Moreover, the structure and properties of the dendrimer and their synthesis approaches are also discussed in brief. A special focus capitalizes on the conjugations strategies employed for the fabrication of the dendrimer based functional coatings on various implantable substrates. The reflection on the functional abilities of dendrimers clearly demonstrates the potential and indeed substantiate the higher hopes for the future of dendrimer based implant coatings.

6.2 Dendrimer

Dendrimers (derived from the *Greek* word "*dendron*" meaning "tree/branch" and "*meros*" meaning "part") are monodispersed, hyper-branched, three-dimensional nanoscale macromolecules possessing high surface group density. Typically, these are composed of three distinct domains (1) multifunctional core, (2) dendrons (branching units), and (3) terminal functional groups (Fig. 6.1). The core moiety is surrounded by the sequentially organized layers of dendrons and with each outgrown layer the number of intrinsic and extrinsic functional groups, branching points and molecular weight increases exponentially. The dendron layers are termed as 'generation' and represented as "Gx", where'x' represents the generation number. The uniform distribution of dendrons around the core results in the formation of a three-dimensional globular structure of the dendrimer with some void spaces in the interior, which are known as 'dendrimeric crevices' or 'dendritic cavity'. The size and shape of the dendrimer molecule depend on its generation and physicochemical

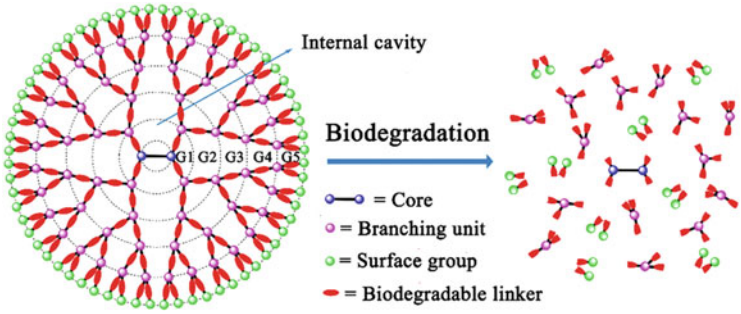

Fig. 6.1 A schematic representation of a dendrimer showing different structural components. Reprinted with the permission from Ref. (Huang and Wu 2018) copyright © Elsevier

properties are on core and dendrons. Typically, with an increase in the generation number the size of dendrimer also increases, and at the same time, these macromolecules undergo a conformational change and tend to become compact. For example, lower generation dendrimers (G1–3) possess an open starfish-shaped and highly asymmetric structure, while the higher generation dendrimer ($\geq$G4) possesses a close, densely packed, and nearly spherical shaped structure.

Typically, the dendrimer is produced by following iterative grafting reactions in a divergent or convergent way. The traditional reactions such as the Staudinger reaction, Michael addition, Sonogashira coupling Williamson etherification, and Mitsunobu esterification, are being utilized for the dendrimer synthesis (Newkome et al. 2001). Some advanced chemical reactions such as orthogonal coupling reaction (Montañez et al. 2010), solid-phase synthesis (Swali et al. 1997), organo-transition-metal chemistry (Casado et al. 1999; Liu and Puddephatt 1996), organo-silicon chemistry (Dvornic et al. 2000), and organo-phosphorus chemistry (Marmillon et al. 2001) are also being used for dendrimer synthesis. The synthesis aspects of dendrimers are already discussed in detail in several invaluable comprehensive reviews (Carlmark et al. 2009; Satija et al. 2011; Tomalia 2005, 2012; Walter and Malkoch 2012), hence, it is not covered in this chapter.

Till date, several families of dendrimers and their conjugates have been utilized for various biomedical applications (Araújo et al. 2018). Among these, the Tomalia-type star-shaped poly (amidoamine) (PAMAM) dendrimer family of the dendrimer is the most widely studied, because of their ease of synthesis, versatility in functionalization, and commercial availability (Tomalia 2005, 2010). Some other commonly used dendrimer families includepoly-L-lysine, melamine, poly (propylene imine), polyether, ferrocene, polyester, peptide, polyaryl, porphyrin, phosphorous, phenylazomethine, glycopeptide dendrimer, and polyglycerol (Nimesh 2013).

Several properties and applications of the dendrimers are associated with the pendant groups, however, the internal functionality of the dendritic cavity, nature of the dendrons and the core also play a very important role. However, the functional versatility of the dendrimers arises not only from their unique combination of intrinsic dendritic and structural properties but also from their physicochemical and biological properties. Most of these inherent properties critically depend on the nanoscale design parameters of the dendrimer (i.e. size, shape, surface chemistry, composition, and flexibility) and this dependency can be defined as 'dendritic effect'(Tomalia 2012). This dendritic effect can be demonstrated by comparing the behavior of a terminal group as an individual molecule and after conjugated with dendrimer. For example, the fluorophore molecules attached to the terminal functional groups of a dendrimer shows brighter fluorescence signal, when compared to their monomeric form due to a significant change in the emission coefficient (Sali et al. 2006).

Various properties of dendrimers have been exploited to develop function-oriented products including implant coatings (Gou et al. 2017), anti-cancer drugs (Kesharwani and Iyer 2015), sensors (Balzani et al. 2000), and gene delivery (Fu et al. 2008), etc. Particularly, multivalent nature, globular shape, dendrimeric cavities and the versatility in the functionality of dendrimer have been of great

importance for the development of biomedical implant coatings. The compact size, hierarchical order of components and multivalent surface have made these macromolecules ideal for developing ultra-thin functional coatings. The following section focuses on various properties of the pristine and engineered dendrimer and their functional applications for the development of various biomedical implant materials, especially for dental and bone implants.

6.3 Strategies of Dendrimer Coating on Implants

The dendrimer coating on implant substrates has been achieved by means of both non-covalent and covalent conjugation methods. The choice of the conjugation method depends upon composition & topography of the implant materials and the types of functional groups present on the dendrimer and implant surface. Till date, dendrimer of different surface functional groups such as carboxyl, amine, hydroxyl, and phosphate, etc. have been successfully coated on hydroxyapatite and titanium-based substrates using various conjugation protocols (Table 6.1). This section highlights the most commonly employed dendrimer coating strategies on dental and bone implants along with their advantages and limitations.

6.3.1 Non-covalent Coating

Non-covalent immobilization techniques are typically based on the nature and type of the peripheral functional groups present on the dendrimer surface and the implant material. The conjugation essentially relies upon the types of interactions that might include physical adsorption, Vander Waals forces, electrostatic (including hydrogen bonding), and hydrophobic interactions (Johnson et al. 2010). Particularly, for dendrimer-based implant coatings development, electrostatic interactions have been employed widely as the cationic or anionic peripheral groups of the dendrimer strongly interact with oppositely charged groups present on the implant surface. For instance, an amine group possessing cationic PAMAM dendrimer can be immobilized on hydroxyl groups (anionic in nature) bearing implant material by means of electrostatic interactions. However, the binding stability of dendrimer film will depend upon multiple factors including pH, solvent medium, the concentration of the dendrimer, functional group density at the implant surface, topography of the implant, and the generation of dendrimer, etc. Till date, numerous types of dendrimer immobilized implant materials have been fabricated for various biomedical applications by employing either the pristine or surface engineered dendrimer. On the basis of types of dendrimers employed for the implant coatings, their coating strategies can be broadly classified in the following two categories.

6.3.1.1 Single Step Pristine Dendrimer Coating

In this approach, the implant substrates are simply incubated with unmodified dendrimer for a specific duration to obtain the dendrimer-functionalized implant.

Table 6.1 Various conjugation strategies employed for the development of dendrimer-coatings on implant

Dendrimer type	Implant type	Characteristics	References
Non-covalent interactions			
SSP-PAMAM-NH_2 (G4)	Hydroxyapatite	• Strong electrostatic interactions between anionic statherin-coated dendrimers and cationic calcium on the implant surface	(Gou et al. 2017)
PAMAM-NH_2 (G4), PAMAM-COOH (G4), PAMAM-OH (G4)	Human dentin	• Electrostatic interactions via calcium coordination complexation by the charged groups on the dendrimer • Size exclusion (6–40 kDa) features of collagen fibrils	(Tao et al. 2017)
PAMAM-COOH (G4)	Human dentin	• Electrostatic interactions between the carboxylic groups of dendrimer and dentin crystals • Retention of dendrimer within the collagen microfibrils	(Zhou et al. 2014)
PAMAM-NH_2 (G3)	Human dentin	• Electrostatic interactions via calcium coordination complexation by the charged groups on the dendrimer • Size exclusion (6–40 kDa) features of collagen fibrils	(Liang et al. 2017; Liang et al. 2016; Liang et al. 2018)
PAMAM-NH_2 (G4)	Human dentin	• Electrostatic interactions via calcium coordination complexation by the charged groups on the dendrimer • Size exclusion (6–40 kDa) features of collagen fibrils	(Bae et al. 2019; Gao et al. 2017; Jia et al. 2014)
PAMAM-NH_2 (G5)	MAO-Ti	• Electrostatic interactions between cationic dendrimer and anionic hydroxyl groups on the MAO-Ti surface	(Wang et al. 2011)
PAMAM-PO_3H_2 (G4)	Tooth enamel	• Electrostatic interactions between phosphate ions of the dendrimer and Ca ions of the enamel	(Chen et al. 2014)
PAMAM-PO_3H_2 (G3–4)	Human dentin	• Electrostatic interactions between phosphate ions of the dendrimer and Ca ions of the enamel • Size exclusion (6–40 kDa) features of collagen fibrils	(Zhu et al. 2018)
PS-PL dendrons (G3)	Ti_6Al_4V	• Physical adsorption by means of spray coating	(Meikle et al. 2013)
ALN-PAMAM-COOH (G5)	Tooth enamel	• Ligand exchange reaction where two phosphate groups of the ALN molecule replace two	(Wu et al. 2013)

(continued)

Table 6.1 (continued)

Dendrimer type	Implant type	Characteristics	References
		surface phosphate groups of HA	
ALN-PAMAM-COOH (G3.5) and star-PDMAEMA	PLA/HA	• Ligand exchange reaction where two phosphate groups of the ALN molecule replace two surface phosphate groups of HA • Layer-by-layer deposition by means of electrostatic interactions between anionic dendrimer and cationic star polymer	(Wu et al. 2015)
PAMAM-COOH (G3.5)	Hydroxyapatite	• Electrostatic interactions via calcium coordination complexation by the charged groups on the dendrimer and hydroxyapatite • Retention of dendrimer within the collagen microfibrils	(Lin et al. 2017)
PAMAM-COOH (G3)	Human dentin	• Electrostatic interactions between phosphate ions of the dendrimer and Ca ions of the enamel • Size exclusion (6–40 kDa) features of collagen fibrils	(Chen et al. 2013; Liang et al. 2019)
PAMAM-COOH (G3.5)	Human dentin	• Electrostatic interactions between phosphate ions of the dendrimer and Ca ions of the enamel • Size exclusion (6–40 kDa) features of collagen fibrils	(Xie et al. 2016)
PS-PL dendrons	ZirTi	• Electrostatic interactions between the charged groups of dendrimer and dentin crystals. • Retention of dendrimer within the collagen microfibrils	(Bengazi et al. 2014; Galli et al. 2014)
PS-PL dendrons (G2)	Ti_6Al_4V	Physical adsorption by means of spray coating	(Stübinger et al. 2015)
PAMAM-PO_3H_2 (G4)	Human dentin	• Electrostatic interactions between phosphate ions of the dendrimer and Ca ions of the enamel • Size exclusion (6–40 kDa) features of collagen fibrils	(Zhang et al. 2015)
Covalent interactions			
NH_2-terminated dendrimers (G1–4)	Si/TiO2	Generational growth of dendrimers on APPA functionalized Ti implant which	(Li et al. 2018)

(continued)

Table 6.1 (continued)

Dendrimer type	Implant type	Characteristics	References
		are covalently linked through silyl groups	
PAMAM-NH_2 (G5) PAMAM-COOH (G5) PAMAM-CH_3 (G5)	Silicon (Si)	• Silyl group covalent binding among amino group of dendrimer and Si on the implant	(Staehlke et al. 2019)

SSP = Statherinphosphorylated PAMAM-COOH = PAMAM-PO_3H_2; PS-PL = Polyserine-Polylysine; MAO-Ti = apatite coated titanium; PLA/HA = Polylactic acid/hydroxyapatite; ZirTi = Zirconia sand blastered acid etched titanium

For example, Chen et al. (2013), reported the formation of a self-assembled monolayer of PAMAM-COOH (G3) dendrimer on acid-etched enamel (obtained from the human tooth) by means of electrostatic interactions (Chen et al. 2013). The binding of dendrimer was based on the fact that PAMAM-COOH dendrimer is anionic in nature, which can electrostatically interact with cationic calcium present on the enamel surface resulting in their self-assembly. By employing a similar strategy, Zhou et al. developed the triclosan (an antibiotic drug) loaded PAMAM-COOH (G4) dendrimer film on ethylenediaminetetraacetic acid (EDTA)-etched human dentin sample to simultaneously cure dental caries and remineralization of the damaged dentin (Zhou et al. 2014). In a recent study, Tao et al. (2017) investigated the immobilization of three different dendrimers, i.e. PAMAM-COOH, PAMAM-OH and PAMAM-NH_2, on demineralized dentin simply by incubating for 1 h (Tao et al. 2017). In all the cases, the dendrimer formed a good film on dentin surface that was ascribed to (1) electrostatic interactions via calcium coordination complexation, wherein the binding affinities were observed in the order of PAMAM-COOH > PAMAM-NH_2 > PAMAM-OH, and (2) the size-exclusion features of collagen fibrils, i.e. the fibrils allowed smaller molecules (6–40 kDa) comparable to the size of the dendrimer to freely pass through the collagen fibrils. By exploiting a similar type of conjugation chemistry, some other research groups have also demonstrated the successful development of dendrimer-coated implant substrates (Liang et al. 2016).

Although the non-covalent conjugation strategies of the native dendrimer are rapid, facile, and user-friendly, the stability of the resulted films, especially under dynamic flow conditions and further for *in-vivo* applications, still remains a challenge. This is because leaching of dendrimer molecules may occur, which eventually fails the purpose of functional coating along with the other possible side effects of free dendrimer molecules, and the exposed implant surface.

6.3.1.2 Multi-Step Surface Engineered Dendrimer Coating

In order to improve the stability of dendrimer coating on implants and to reduce the toxicity of the pristine dendrimers, a few research groups have proposed the use of surface-engineered dendrimers. These approaches involve two or more number of steps, where at first the pristine dendrimer is modified with certain functional molecules such as polyethyleneglycol (PEG), peptides, and chemical linkers etc.

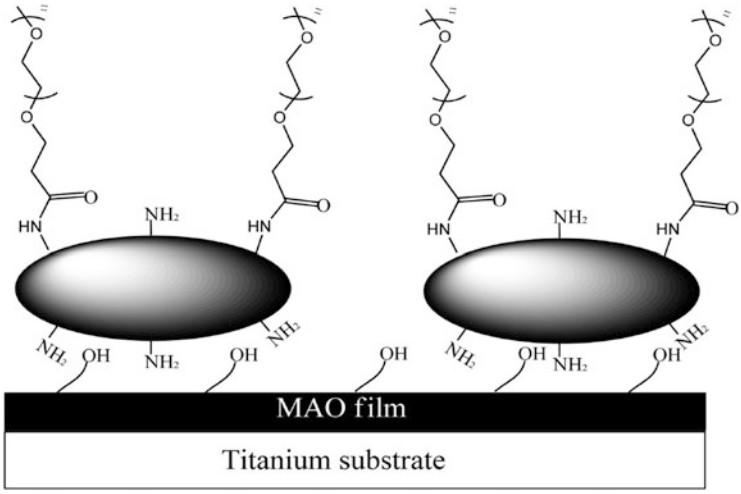

Fig. 6.2 Schematic representation of PEGylated PAMAM dendrimer film on Micro-arc oxidized-titanium (MAO-Ti). Reprinted with the permission from Ref. (Wang et al. 2011) copyright © American Chemical Society

followed by their noncovalent conjugation with the implant materials. For example, Wang et al. (2011), developed the coating of partially PEGylated PAMAM-NH_2 (G5) dendrimer on micro-arc oxidized titanium (MAO-Ti) substrate (Wang et al. 2011). The PEG molecules are well-known for their high hydrophilicity and biocompatibility, and the PEGylation of dendrimer helps in silencing the cationic amine group of the PAMAM dendrimer and reduces their cytotoxicity towards mammalian cells (Lopez et al. 2009; Satija et al. 2007). The MAO process on titanium substrate istypically carried out to incorporate the apatite (i.e. calcium phosphate) layer, which can serve as a barrier type structure inside and a porous structure outside (Cheng et al. 2012; Das et al. 2007; Park et al. 2007). This can also facilitate the inward growth and bonding between the osseous tissue and implant. Upon incubation of the partially PEGylated PAMAM-NH_2 dendrimer with MAO-Ti substrate, the modified dendrimer formed a stable monolayer by means of (1) electrostatic interactions among the cationic amine groups of dendrimer and anionic hydroxyl groups on the MAO-Ti surface, and (2) the complexation of amino groups of the dendrimer with Ti^{4+} ions of implant (Fig. 6.2).

The peptide molecules have also been used to design a dendrimer-decorated implant substrate, which can enhance the conjugation strength and eventually stabilize the dendrimer film on the implant surface. For example, Gou et al. (2017), have suggested the use of statherin (SSP) (an acidic phosphoprotein) peptide-functionalized dendrimer to develop the functional coating on hydroxyapatite (HA) surface (Gou et al. 2017). The SSP peptide has been demonstrated to specifically recognize and strongly adsorb on HA surface, as its first six aminoacids (DpSpSEEK) are highly negatively charged that form ionic complexes with calcium ions present on HA (Raj et al. 1992). To obtain the SSP-dendrimer conjugate coated

HA, first SSP was interacted with PAMAM-NH_2 (G3) dendrimer, which yielded the statherin-dendrimer (SSP-PAMAM-NH_2) conjugatedue to electrostatic interactions between the cationic amine groups of the dendrimer and negatively charged tyrosine groups of peptide. Subsequently, the SSP-PAMAM-NH_2 conjugate was immobilized on the HA substrate by means of drop-casting followed by gentle washing with PBS buffer. The authors suggested that the SSP-PAMAM-NH_2 conjugate strongly adsorbs on the HA surface by means of specific strong electrostatic interactions among the calcium ions, present on the HA surface, and the carboxylic groups (anionic) of the SSP peptide.

Since dental and bone remineralization requires the significant adsorption of calcium ions on the implants along with other essential biochemicals, few research groups have suggested the use of phosphorous-containing dendrimers. Chen et al. (2014), demonstrated the use of phosphate-modified PAMAM dendrimer (G4), i.e. PAMAM-PO_3H_2 for HA coating (Chen et al. 2014). There were two main reasons for modifying the dendrimer with phosphate moieties: (1) the phosphate groups have stronger affinity for calcium ions and therefore, they can provide strong binding towards HA surface, and (2) they can act as analogs of amelogenin (i.e. a protein involved in the mineralization of enamel to form a highly organized matrix of rods, inter-rod crystal, and protein). The PAMAM-PO_3H_2 dendrimers could be successfully adsorbed on the acid-etched enamel surface by pipetting on to the window side of the tooth enamel surface, demonstrated for the regeneration of human tooth enamel in artificial saliva. A higher adsorption capability was attributed to the terminal phosphate group, which can form Ca coordination bonds (stronger interlinking). By following a similar strategy, Zhu et al. (2018) developed the coating of apigenin (API) encapsulated phosphorylated PAMAM (G3 and G4) dendrimers on demineralized dentin (Zhu et al. 2018). The phosphate-terminated dendrimer was synthesized by modifying the PAMAM-NH_2 with ethyldimethylaminopropylcarbodiimide (EDC) and N-hydroxy-2,5-pyrrilidinedione (NHS), followed by interaction with 3-phosphonopropionic acid. These anionic phosphorylated PAMAM-NH_2 dendrimer formed a self-assembled monolayer on the cationic dentin surface via electrostatic interactions.

Phosphoserine (PS) is known as an effective functional moiety for inducing bio-mineralization in living tissues (Reinstorf et al. 2004). The phosphate amino acid (i.e. PS) interacts strongly with calcium ions and catalyzes the formation of apatite crystals. The biomineralization and osteoconductive property of PS have been exploited by Meikle et al. (2013) for coating the titanium-based substrate with phosphoserine-modified poly (ε-lysine) (PS-PL) dendrons (Meikle et al. 2013). The solid-phase synthesis of PS-PL dendrons was carried out in two steps: (1) first, the G3-PL dendrons conjugate was prepared by adding Fmoc-Lys(Fmoc)-OH molecules to the core moiety (i.e. glycine) sequentially, and (2) then terminating the process using Fmoc-Ser(PO(OBzl)OH)-OH. In this synthetic process, the carboxylic groups on amino acid were activated at each step using O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate as coupling agent, whereas amino group could be exposed by cleaving the Fmoc protecting group with piperidine (prepared in dimethylformamide). After deprotection of the PS groups, PS-PL dendrons were cleaved from the resin and used after purification. The

PS-PL dendrons were immobilized on the acid-etched titanium alloy (Ti_6Al_4V) via a spray coating technique, yielding a nanostructured surface with textured surface morphology. The spray coating technique showed relatively stronger bonding, when compared with the dip coated one, which usually results in dendrimer films of varying thickness with poor reproducibility.

Alendronate (ALN) is known to adsorb on the HA surface by a ligand exchange reaction between the phosphate groups of ALN and HA (Palazzo et al. 2007). This property of the ALN has been exploited for remineralization of the tooth by employing the ALN-modified PAMAM-COOH dendrimer (Wu et al. 2013). The ALN-modified PAMAM-COOH dendrimer (G3.5) dendrimer was prepared by means of carbodiimide coupling chemistry and subsequently drop-casted on tooth enamel surface to yield a stable dendrimer film (1.6 ALN per dendrimer molecule). The presence of ALN showed greater binding strength and loading efficiency (20–25% higher) of ALN-PAMAM-COOH towards HA compared to the PAMAM-COOH dendrimer alone. Later, the same research group proposed the strategy to developed a multilayer film of pH-sensitive star polymer and ALN-PAMAM-COOH dendrimer (loaded with indomethacin, an anti-inflammatory drug) on PLA-HA composite material (Wu et al. 2015). In this approach, first, the ALN-PAMAM-COOH conjugates were immobilized on the PLA-HA substrate followed by incubating the ALN-PAMAM-COOH-PLA-HA substrate with cationic star-poly[2-(dimethylamino)ethylmethacrylate] (star-PDMAEMA) polymer to develop ALN-PAMAM-COOH-star-PDMAEMA (Fig. 6.3). Further, for multilayer coating on the PLA-HA substrate, six alternative cycles of the layer-by-layer deposition of indomethacin (IND) drug-loaded ALN-PAMAM-COOH (anionic) and star-PDMAEMA polymer were repeated. The developed ALN-PAMAM-COOH-star-PDMAEMA composite film demonstrated the potential as a self-anti-inflammatory biodegradable implant material.

Several studies have revealed that the incorporation of growth factors, such as fibroblast growth factor (FGFs), bone morphogenetic protein-2(BMP2), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and epidermal growth factor (EPG) can promote bone healing and improve osseointegration (Alam et al. 2007; Nandi et al. 2010). However, the issues associated with transient activity and easy proteolytic degradation, due to inefficient burst release, causes the slow and limited formation of the new bones. To minimize these variations, Chen et al. (2018) suggested the localized and prolonged delivery of functional gene coding the growth factors. In this study, the cationic PAMAM-NH_2 (G5) dendrimers were conjugated with anionic plasmid DNA (hBMP2 gene containing DNA and EGFP marker) to form DNA-PAMAM (d-DNA) conjugate by means of electrostatic interactions. Further, the developed cationic conjugate (due to free amine groups) was grafted on the anionic titanium implant (pre-coated with polyethyleneimine and hyaluronic acid) via electrostatic interactions. Thereafter, a layer of naked plasmid DNA was conjugated electrostatically to form a d-DNA/DNA bilayer. These coatings were repeated four times with each of the d-DNA and naked plasmid DNA, which were electrostatically stable due to interactions between polyanions and polycations. The resulted composite film $(\text{d-DNA/DNA})_4$ on the implant material yielded the thickness of about 16–60 nm.

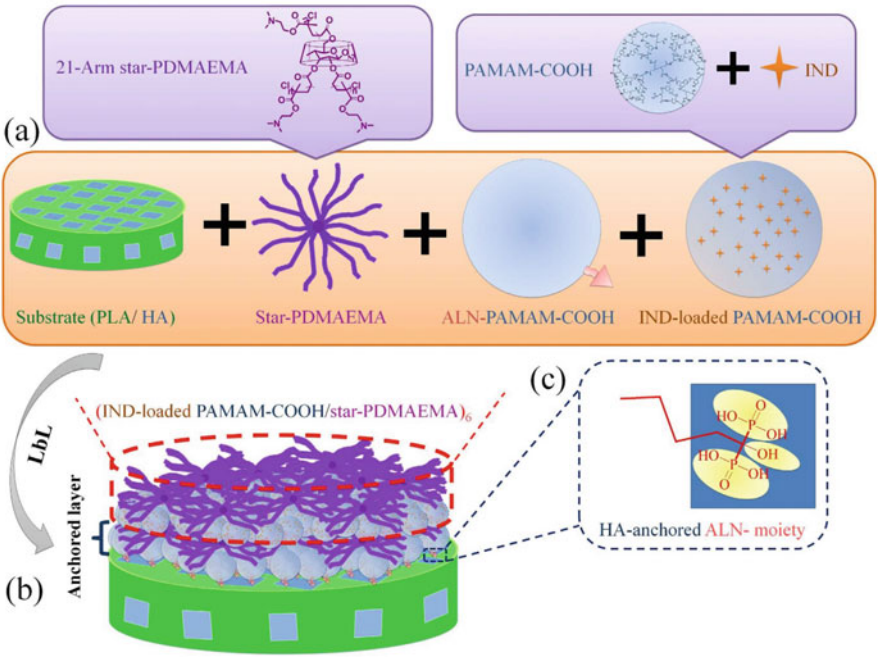

Fig. 6.3 Schematic illustration of the substrate-anchored and degradation-sensitive anti-inflammatory coating for implant materials: (**a**) Biodegradable substrate of implant material (PLA/HA) and building blocks of the LbL coating (star-PDMAEMA, ALN-PAMAM-COOH and IND-loaded PAMAM-COOH); (**b**) Alendronate modified dendrimer-indomethacin conjugate (Coating-AP/S) in the form of (**c**) Substrate-anchored property provided by the ALN moiety. Reprinted with the permission from Ref. (Wu et al. 2015) copyright © Springer Nature

6.3.2 Covalent Coating

The covalent conjugation approach is well-known to generate stable coatings that can offer the desired characteristics relatively for longer period of time. Numerous types of covalent conjugation chemistries are being explored in the literature (Renault et al. 2018; Sunasee and Narain 2014; Wickramathilaka and Tao 2019). However, only few strategies have been employed to develop covalent conjugation chemistry based dendrimer coatings on implant surfaces. Li et al. (2018) reported the in-situ fabrication of dendrimer coated Ti substrate, where Ti substrate was activated by functionalizing it with 3-aminopropyl phosphonic acid (APPA) to generate the hydroxyl groups (Li et al. 2018). Thereafter, the amine-terminated dendrimers were grown *in-situ* on TiO_2-APPA substrates by generational growth by following the aminolysis and Michael addition reaction (Naidu et al. 2003) (Fig. 6.4).

In another study, Staehlke et al. (2019) reported the self-assembly of dendrimers on SiO_2 model implant substrate (Staehlke et al. 2019). In the first step, the SiO_2 substrates were modified with 3-(triethoxysilyl)propyl succinic acid anhydride to generate the anhydride groups, which were subsequently coupled with PAMAM-

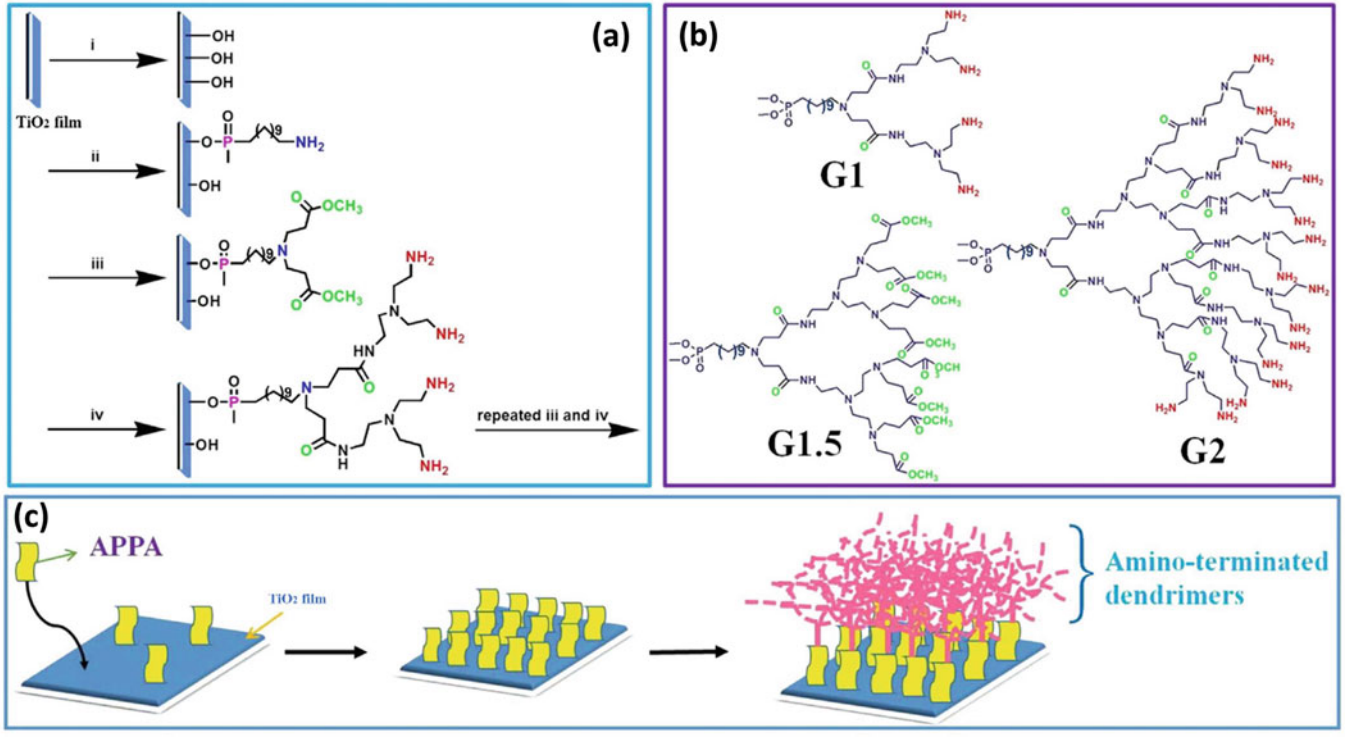

Fig. 6.4 Schematic representation of synthesis of amino-terminated dendrimers: (**a**) amino-terminated dendrimer construction: (i) Sulfuric acid/hydrogen peroxide (H_2SO_4/H_2O_2), (ii) 3-aminopropyl phosphonic acid (APPA), (iii) Methyl acrylate (MA), and (iv)tris-(2-aminoethyl)-amine (TAEA). (**b**) Structure of G1-NH_2, G1.5-NH_2, and G2-NH_2 amino-terminated dendrimers. (**c**) Cartoon illustrating the growth of amino-terminated dendrimers on TiO_2 film. Reprinted with the permission from Ref. (Li et al. 2018) copyright © Elsevier

NH_2 dendrimer (G5) at room temperature as the anhydride groups are very reactive towards the nucleophilic amine groups containing molecules. Thereafter, the terminal amine groups were transformed into either carboxylic acid groups (PAMAM-COOH) or methyl groups (PAMAM-CH_3) by reacting with succinicanhydride or acetic acid anhydride, respectively (Fig. 6.5).

6.4 Applications of Dendrimer-Coated Implants

6.4.1 Biomineralization

Biomineralization is a life long, complex, dynamic process by which living organisms form the certain inorganic nanoscale crystals for different functional purpose such as bone development, tooth formation, mechanical stiffening of tissue, magnetic or gravitational sensing, and element storage, etc. (Gorski 2011; Veis and Sabsay 1983). Among the different nanocrystals, HA is one of the most important inorganic nanocrystal for human beings as it constitutes the bone (up to 50%) and tooth (70–80%) providing the strength and rigidity (Habibah 2018). In course of lifetime, the bones and teeth are at risk of demineralization, because of their location and anatomical arrangement, where they are exposed to consumables and microbiota. The demineralization reduces the rigidity of these tissues, resulting in the formation of dental caries and fractures, which eventually causes pain. Therefore, remineralization of the affected bone and tooth is very important to maintain their natural integrity (Abou Neel et al. 2016). The remineralization can be artificially achieved by utilizing the implants either mimicking the properties of natural HA or by facilitating the HA formation that are equally or more efficient and sustainable at the implanted site of interest.

In order to improve the biomineralization of bone and tooth, numerous strategies have been reported including the use of probiotic bacteria, fluoride therapy, apatite cements, and brushite cement, etc. (Abou Neel et al. 2016; Jensen et al. 2012; Ramanujam et al. 2019). The dendrimer-coated implants have also shown efficient biomineralization process, which are primarily associated with their structural and dendritic properties (Table 6.2). For example, Chen et al. (2013) investigated the regeneration of HA on etched human enamel surface after coating with anionic PAMAM-COOH dendrimer. After immersing the PAMAM-COOH-engineered enamel in the calcium phosphorous solution for 20 h (under near-clinical conditions), well-organized, rod-shape HA crystals were obtained, which were found to be arranged parallel to the long axis of enamel crystals (Fig. 6.6a). On the other hand, in the absence of the dendrimer film, irregularly arranged flake-like HA crystals with large interspacing were formed (Fig. 6.6b). Authors suggested that the presence of PAMAM-COOH dendrimer on enamel surface that served as the template for facilitating the nucleation of calcium ions by means of electrostatic interactions due to the presence of the anionic carboxyl groups. This might have led to increase in the local concentration of Ca^{2+} ions, which subsequently facilitated the formation and deposition of HA in the presence of phosphate ions. As the deposition

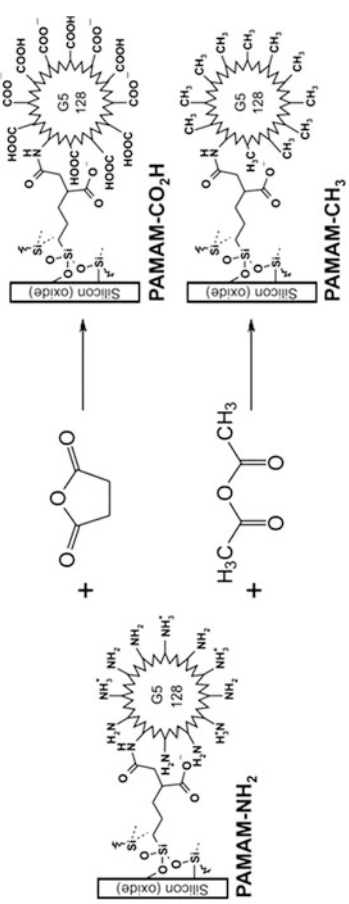

Fig. 6.5 Formation of surface coatings via G5-dendrimers on silicon substrate with terminal amino (NH_2), carboxylic acid (COOH) and methyl (CH_3) groups. Reprinted with the permission from Ref. (Staehlke et al. 2019) copyright © Elsevier

Table 6.2 Comprehensive overview of functional advantages of dendrimer-functionalized implants

Dendrimer type	Implant substrate type	Functional roles	References
SSP-PAMAM-NH_2 (G4)	Hydroxyapatite	• Antibacterial activity against *S. aureus*, *E. coli, S. sanguinis and F. nucleatum* • Biocompatible with osteoblastic MC3T3-E1 cells	(Gou et al. 2017)
PAMAM-COOH (G3)	Tooth enamel	• Rod-like HA crystals formation • Remineralization in 20 h	(Chen et al. 2013)
PAMAM-NH_2 (G4), PAMAM-COOH (G4), PAMAM-OH (G4)	Human dentin	• Biomineralization in 28 days	(Tao et al. 2017)
PAMAM-NH_2 (G3)	Human dentin	• Needle-like crystal structure on the dentin surface and in the dental occlusions • Biomineralization in 21 days • Ca/P = 2 • Hardness = 0.53 ± 0.04 GPa	(Liang et al. 2016)
PAMAM-NH_2 (G5)	MAO-Ti	• Antibacterial activity against *P. aeruginosa and S. aureus* • Biocompatible with osteoblastic hMSCs (human bone mesenchymal stem cells)	(Wang et al. 2011)
PAMAM-PO_3H_2 (G4)	Tooth enamel	• Rod-like crystal formation • Biomineralization in 3 weeks • Biocompatible with L929 cells	(Chen et al. 2014)
PAMAM-PO_3H_2 (G3–4)	Human dentin	• Needle-like crystal structure formation • Biomineralization in 4 weeks • Ca/P = 1.69 and 1.74 • Antibacterial action against *S. mutans*	(Zhu et al. 2018)
PS-PL dendrons (G3)	Ti_6Al_4V	• Osseointegration of MG-63 and SAOS-2 osteoblast-like cells (up to 72 h)	(Meikle et al. 2013)
ALN-PAMAM-COOH (G5)	Tooth enamel	• Rod like crystal of uniform shape and size formation • Biomineralization in 4 weeks • Ca/P = 1.670 • Biocompatible with osteoblastic HepG2 cells	(Wu et al. 2013)
ALN-PAMAM-COOH (G3.5) and star-PDMAEMA	PLA/HA (10% HA)	• Indomethacin loaded dendrimer • Significant reduction in levels of inflammatory cytokines, i.e., IL-1 β, IL-6, and TNF-α compared to uncoated implants • Anti-inflammatory action in 8 weeks	(Wu et al. 2015)

(continued)

Table 6.2 (continued)

Dendrimer type	Implant substrate type	Functional roles	References
NH_2-terminated dendrimers (G1–4)	TiO_2	• Anti-coagulant • Biocompatible with HUVEC cells • Osseointegration of HUVEC cells (3 days)	(Li et al. 2018)
PAMAM-NH_2 (G5), PAMAM-COOH (G5), PAMAM-CH_3 (G5)	Silicon	• Osseointegration of MG-63 osteoblast cells in 24 h	(Staehlke et al. 2019)
PAMAM-COOH (G4)	Human dentin	• Flake-like structure formation • Biomineralization in 4 weeks • Ca/P = 1.63 • Biocompatible with L929 cells	(Zhou et al. 2014)
PAMAM-NH_2 (G3)	Human dentin	• Needle-like crystal structure formation on the dentin surface and in the dental occlusions • Biomineralization in 20 days • Ca/P = 1.3 • Dentin hardness = 0.43 ± 0.02 GPa	(Liang et al. 2017)
PAMAM-NH_2 (G3)	Human dentin	• Needle-like crystal structure formation on the dentin surface and in the dental occlusions • Biomineralization in 42 days	(Liang et al. 2018)
PAMAM-NH_2 (G4)	Human dentin	• Needle-like crystal formation • Biomineralization in 28 days • Deposition in dental occlusions	(Gao et al. 2017)
PAMAM-NH_2 (G4)	Human dentin	• Sphere-shaped mineral deposition • Biomineralization in 30 days	(Bae et al. 2019)
PAMAM-NH_2 (G4)	Human dentin	• Distinct, flat crystal layer-like deposition covering the dental occlusion • Biomineralization in 8 weeks • Microhardness = 98.69 ± 3.359 HV	(Jia et al. 2014)
PAMAM-COOH (G3.5)	Human dentin	• Biomineralization in 7 days • Needle-like crystal formation • Microhardness = 43.26 ± 5.40 HV	(Lin et al. 2017)
PAMAM-COOH (G3)	Human dentin	• Needle-like crystal structure formation on the dentin surface and in the dental occlusions • Biomineralization in 35 days • Ca/P = 1.6 • Dentin hardness = 0.52 GPa	(Liang et al. 2019)

(continued)

Table 6.2 (continued)

Dendrimer type	Implant substrate type	Functional roles	References
PAMAM-COOH (G3.5)	Human dentin	• Flake-like crystal formation • Biomineralization in 6 weeks • Ca/P = 1.71	(Xie et al. 2016)
PS-PL dendrons	ZrTi	• Osseointegration of cells surrounding the implant material	(Bengazi et al. 2014)
PS-PL dendrons (G3)	ZrTi	• Osseointegration of MC3T3-E1 cells in 10 days • Biocompatible with MC3T3-E1 cells	(Galli et al. 2014)
PS-PL dendrons (G2)	Ti_6Al_4V	• Osseointegration in sheep bone cells within 8 weeks	(Stübinger et al. 2015)
PAMAM-PO_3H_2 (G4)	Human dentin	• Deposition of crystals both on the surface of dentin and in the dentinal tubule • Biomineralization in 4 weeks • Ca/P = 1.675 • Biocompatible with HepG2 cells	(Zhang et al. 2015)
PAMAM-NH_2 (G3)	Human dentin	• Needle-like crystal formation • Biomineralization in 21 days • Deposition in dental occlusions	(Xiao et al. 2017)

SSP = Statherin; Phosphorylated PAMAM-COOH = PAMAM-PO_3H_2; PS-PL = Polyserine-Polylysine; MAO-Ti = apatite coated titanium; ZirTi = zirconia sand blastered, acid etched titanium

continues, the rod-like HA crystals start growing at specific sites along the long axis of enamel crystals.

In a similar approach, Liang et al. (2014) examined the effect of PAMAM-OH (G2 and G4) dendrimers coating on remineralization of dentin. Upon incubation of the dendrimer-coated implants in the artificial saliva for 21 days, needle-like crystalline minerals, resembling the intact dentin, were formed in the dental occlusions. This was due to the fact that the anionic hydroxyl groups of the dendrimer acted as nucleation site for Ca and P adsorption, which led to calcium phosphate deposition in the occlusions. The G4 PAMAM-OH coated implants showed higher Ca and P percentage value (18.22% and 10.99% for Ca and P, respectively) than the bare implants, i.e. without dendrimer coating, (6.70% and 3.08% for Ca and P, respectively). In the bare dentin samples, all dental occlusions were slightly deposited with minerals, which might be because of the remnant apatite seeds that could have act as nucleation templates for Ca and P adsorption. Compared to G2 PAMAM-OH dendrimer (MW = 3272 kDa), G4 dendrimer (MW = 14,279 kDa) showed higher capability of remineralization, which was ascribed to (1) the presence of greater number of anionic –OH functionalities on the surface, leading to better interaction between the implant and dendrimer and (2) the size-exclusion features of collagen fibrils, i.e. the fibrils allow molecules, ranging from 6–40 kDa, to pass through freely inside the collagen fibrils and facilitate the crystal development.

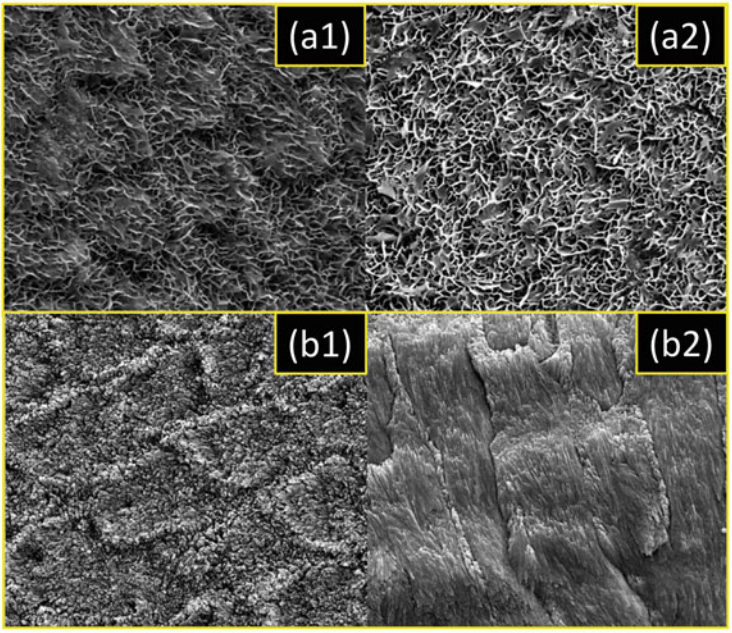

Fig. 6.6 SEM images of acid etched enamel surface. (**a**) SEM image of crystal growth on enamel surface (20 h) without PAMAM-COOH dendrimer and (**b**) with PAMAM-COOH dendrimer Reprinted with the permission from Ref. (Chen et al. 2013) copyright © Elsevier

In the same line, Tao et al. (2017) investigated the effect of dendrimer terminal groups on remineralization of dentin. Upon incubation of the implants in artificial saliva for 28 days, it was found that all the dendrimer, i.e. PAMAM-NH_2, PAMAM-COOH and PAMAM-OH, film coated substrates facilitated the formation of HA on the implant surface, when compared to the uncoated implants, which showed only a little deposition due to residue apatite seed. The dendrimer-coated implants showed an increase in the hardness value by 0.59 ± 0.03 GPa, 0.62 ± 0.05 GPa, and 0.65 ± 0.02 GPa for PAMAM-OH, PAMAM-COOH, and PAMAM-NH_2, respectively. On the other hand, the uncoated implant did not show any improvement in the hardness value. Among the three different dendrimer investigated, the rate of mineralization was observed to be higher in case of PAMAM-NH_2 (82.18 ± 2.96%) and PAMAM-COOH (76.34 ± 4.53%) than the PAMAM-OH (56.30 ± 8.31%) dendrimer. This can be attributed to variations in their electrostatic binding ability to the demineralized collagen fibrils, which is in the order of PAMAM-COOH > PAMAM-NH_2 > PAMAM-OH (Wang et al. 2001).

In a different approach, Wu et al. (2013) used ALN-PAMAM-COOH dendrimer coating on dental implant, which upon mineralization (up to 4 weeks) in the artificial saliva yield rod-like HA crystal growth of uniform size and shape indicating the formation of intact enamel. On the other hand, the control samples, i.e. the

non-coated implants and only PAMAM-COOH dendrimer coated implants, showed the flake-like and irregular blasting cluster like crystal growth, after 1 week of incubation, which was not consistent with the natural human tooth enamel. The micro-hardness of the ALN-PAMAM-COOH-coated implant was observed to be improved (95.5% of the original value of natural enamel), and showed a deposition of more than 10 μm thickness of HA (after 4 weeks), whereas the uncoated implant was able to restore the micro-hardness to a very limited degree. The implantation of the ALN-PAMAM-COOH dendrimer coated implants in the oral cavity of rats showed the induction of HA remineralization on the implant surfaces with better surface morphology after 28 days. On the other hand, cracks were formed in the uncoated and PAMAM-COOH dendrimer coated implants, which might be ascribed to the mechanical stress exerted by the rat during eating and reduce hardness of the grown HA crystals. The Ca/P ratio was found to be 1.670 for ALN-PAMAM-COOH dendrimer coated dental implant, which is similar to natural HA (Ca/P 1.67), whereas the Ca/P ratio were found to be 1.812 and 1.506 for bare and PAMAM-COOH coated dental implants, respectively. In a similar way, Zhou et al. (2014) demonstrated the use of triclosan-loaded PAMAM-COOH (G4) dendrimer derived coatings yielding the crystals resembling the natural HA in terms of size, shape, and degree of orientation.

By inspiring from the natural mineralization phenomenon, the phosphate moiety containing dendrimers have also been investigated to facilitate the remineralization process. For example, phosphate group terminated dendrimer (PAMAM-PO_3H_2) (G4) showed remineralization of HA (prism-like structure) on the implant surface upon incubation in artificial saliva within 3 weeks of time (Chen et al. 2014). Authors reported that the PAMAM-PO_3H_2 dendrimer acts as an analog of amelogenin, which induces the remineralization by attracting Ca and P from the surrounding and depositing it as calcium phosphate on the implant surface. On the other hand, the non-coated implants showed certain amount of HA deposition by means of charge adsorption effect among the free mineral ions and surface charge on the implant. The thickness of deposition was found out to be 11.23 μm in case of PAMAM-PO_3H_2coated implant compared to 6.02 μm in PAMAM-COOH immobilized implant. The surface micro-hardness recovery percentage (%SMHR) of PAMAM-PO_3H_2 coated implants was found to be 97% and an adsorption force of 50 N, which is comparable with previous study (ALN-PAMAM-COOH with 95.5% SMHR and 47 N adsorption force). Further, the *in-vivo* experiments in rat's oral cavity corroborated the in-vitro results and higher degree of HA regeneration was found in case of PAMAM-PO_3H_2 coated implants compared to non-coated implant and PAMAM-COOH coated implant. This higher regeneration led to higher rigidity of the implant, which can resist the most mechanical forces exerted by the animal and prevent cracks formation in the implant material. In contrary, the non-coated implants showed multiple cracks in the implant material, which was due to its poor regeneration ability and lower hardness value. In a similar fashion, Zhu et al. (2018) investigated the potential of phosphorylated PAMAM dendrimer loaded with apigenin (PD-API) on dental implants, which formed the needle-like HA crystals with Ca/P ratio of 1.69 to 1.74 for G3 and G4 dendrimer, respectively. A 1-week

incubation of the coated implants decreased the diameter of dental tubules from original 2 μm to 1.6 μm for PD-G3-API coated implant and to 1.0 for PD-G4-API coated implant. After 4 weeks of incubation the diameter became 0.5 μm and 0.2 μm for PD-G3@API and PD-G4@API implant, respectively, due to deposition of minerals needle-like crystals in the tubules.

Bioactive glass is known to release calcium, phosphate, and sodium ions, which can expedite the mineralization process. Recently, Bae et al. (2019) utilized the PAMAM-NH_2 (G4) dendrimer and mesoporous bioactive glass nanoparticles (MBN) conjugate (PAMAM-MBN) to develop a bioactive film on demineralized dentin to cure dentin hypersensitivity. The composite material coatings successfully sealed (87.86 ± 5.62%) the dentinal tubules within 30 days upon incubation in the simulated body fluid, which was relatively better than the MBN alone (80.35 ± 8.89%). This was due to ions releasing ability of the bioactive glass and the higher ions adsorption capability of the dendrimer due to their multidentate nature.

6.4.2 Antibacterial

Despite of having highly antiseptic operative procedures in the course of an implant surgery, a considerable number of post-operative infections arise. The main reasons for these infections are the adhesion of bacteria on to the implant surface and their subsequent biofilm formation. These post-operational infections may lead to serious complications such as implant rejection resulting in increase in the number of patients requiring implants (like artificial bones and joints). Therefore, the requirement offull proof, infection-free biocompatible implantable substrate is of primary need. The implant coatings developed using dendrimers have shown considerable reduction in the chances of bacterial infection by adhering and destroying the bacterial cells present on the implant surface non-specifically mainly due to their cationic nature. For example, Gou et al. (2017) investigated the antibacterial activity of the SSP-PAMAM-NH_2dendrimer film coated on dental implant. The dendrimer film showed a higher level of biofilm depletion potential against *Staphylococcus aureus*, *Escherichia coli, Staphylococcus sanguinis and Fusobacterium nucleatum* bacteria within 24 h of incubation under standard conditions. The high bactericidal activity of the dendrimer film was due to the presence of multiple primary amine groups at the dendrimer periphery, which acted as (1) non-specific receptor for the bacterial cells due to their anionic nature and (2) bactericidal agent by forming the nano-size pores in cell-wall resulting in the leakage of cell contents and eventually leading to death. Further, in-vivo study, no bacterial infection or biofilm formation was observed even after 4 weeks, when the SSP-PAMAM-NH_2 dendrimer-coated implant was grafted in the rats. This demonstrated that the dendrimer film is highly stable and functionally active even in the in-vivo conditions.

The PEGylated dendrimer coated titanium implants have also shown higher antibacterial activity towards *Pseudomonas aeruginos* a and *Staphylococcus aureus* (Wang et al. 2011). In this study, it was found that higher bacterial destruction

occurred in the non-PEGylated (PAMAM-NH_2) dendrimer and 10% PEGylated PAMAM-NH_2 dendrimer coatings because of the presence of a greater number of free amine groups at the periphery of the dendrimer. A further increase in the PEGylation level reduced the antibacterial activity due to neutralization of the cationic amine groups by PEG. Further, the coatings were found more effective against Gram negative bacteria *P aeruginosa* than the Gram positive bacteria *Staphylococcus aureus* due to the differences in their cell wall composition. The bacterial action (on *Pseudomonas aeruginosa)* of dendrimer on biphasic calcium phosphate/Ti nanocomposite on titanium (BCP-Ti) and pristine titanium was also compared. This inferred that, after 3 h of incubation with bacteria, the bacterial viability was decreased by 62% (PAMAM coated pristine titanium) and 83% (PAMAM coated BCP-Ti) compared to unmodified substrate. In a recent study, Zhu et al. (2018) investigated the effect of PD-API dendrimer coatings on biofilm inhibition capability on dental implants using *Streptococcus mutans*. The ratio between the dead and live bacteria was found to be 1.34 and 1.37 for PD-API (G3) and PD-API (G4) dendrimer coated HA discs, respectively. Which was nearly two-fold higher than the PD (G3) and PD (G4) dendrimer-based coatings alone.

6.4.3 Osseointegration

One of the important prerequisites of the implanted materials is the ability to attract cells on its surface so that the proliferation of the cells can occur (i.e. tissue repairing) which can induce the accelerated bone healing and osseointegration. The adhesion of the cells at implant surface is dictated by the biocompatibility, topography, composition and bioactivity of the implant material (Ratner 2015). Dendrimer-based functional coatings have demonstrated most of these desirable characteristics, which varies as a function of dendrimer generation and level of their surface modification. For instance, Li et al. (2018) investigated the blood compatibility of dendrimer coated titanium substrates, which revealed that the higher generation dendrimer lead to coagulation of the platelets compared to the lower generation ones. The lower generation amine groups-containing dendrimer, up to G3, showed better anti-coagulant activity compared to G4 dendrimer, which was due to their small size and relatively low amine groups surface density. Good cell viability (in human umbilical vein endothelial cells) was observed on TiO_2-APPA and dendrimer coated implants after 1 day of incubation, due to better cell adhesion. After 3 days of incubation, a decrease in cell viability was observed at higher dendrimer generation (G4) on TiO_2 film and TiO_2-APPA film because of the higher cationic group present on the dendrimer (amidogens), lead to bursting of the cell membrane. In an alternative method, Meikle et al. (2013) utilized phosphoserine-modified poly(epsilon-lysine) dendrons coating on titanium implant. The *in-vitro* studies on osteosarcoma MG-63 and SAOS-2 osteoblast cells proved that dendrons coated implant are better substrates in terms of cell adhesion and organization of cells in the cytoskeleton compared to uncoated implant, which ended up in cluster formation.

In a recent study, Staehlke et al. (2019) reported that self-assembled dendrimers on the silicon implant can promote higher cell adhesion. The in vitro studies with osteoblastic MG-63 cell lines inferred that PAMAM-NH_2 coated implant favors higher amount of cell adhesion (three-fold increased cell area) with well-spread cell shape and a close-fitting morphology on its surface compared to PAMAM-COOH and PAMAM-CH_3 coatings due to its hydrophilic characteristics along with positive charge of the dendrimer. On the other hand, PAMAM-COOH and PAMAM-CH_3 dendrimer derived film demonstrated the impairment of morphology and adhesion and eventually inducing the apoptosis of osteoblasts. This was attributed to inhibition of actin cytoskeleton organization which showed diffuse, centrally organized actin fibers and light, single fiber in the cortical front rather than imparting fine fibers in the entire cell body.

6.4.4 Anti-Inflammatory

The implantation procedure is often associated with inflammatory responses, which depends upon the number of factors including the composition, form and topography of the implants. The implant is recognized as a foreign substrate by the immune system of our body and thus induces acute to chronic inflammations. These inflammatory reactions lead to the activation of immune cells which release the chemical mediators, i.e. IL-4 and IL-13. Consequently, these mediators induce the fusion of monocyte/macrophage to form into foreign body giant cells. In order to reduce these inflammatory responses, functional coatings, possessing anti-inflammatory activity, have garnered huge attention recently. For example, Wu et al. (2015) utilized indomethacin (IND), an anti-inflammatory drug, loaded dendrimer as functional coating on PLA-containing HA substrate. After 8 weeks of the subcutaneous implantation of these implants in the male Sprague-Dawley rats, lowest inflammatory reaction was observed in relation with number of macrophages and lymphocytes. The fibrous capsule thickness was also found to be lowest (103.34 ± 2.35 μm) in case ALN-modified dendrimer compared to the dendrimer alone (216.29 ± 4.58 μm). This inferred that the strong interaction of ALN towards HA caused the longer attachment of the dendrimer, which eventually made the release of IND for a longer period of time. This demonstrated that the drug-loaded dendrimer-derived functional coating can effectively minimize the inflammatory response. In a similar way, Zhou et al. (2014) utilized triclosan-loaded PAMAM-COOH (G4) dendrimer as a coating material over the acid etched dentin. The drug release resulted in sustained manner without burst release (32 h) which after 48 h, resulted in sustained drug release.

6.5 Summary and Future Prospective

Functional coatings on the implant material have been investigated recently to minimize the implant failure in future via improving the integrity and natural functioning of cell and aid in greater success with higher sustainability. This happens because of the action of the coated material, which can enhance the implant properties. Although numerous compounds have been explored to develop functional coatings for implant, dendrimers have attained a significant attention as a multifunctional coating agent mainly for dental and bone implants. This is due to the globular shape, multivalent nature, presence of dendrimeric cavity and the functional versatility of dendrimers which allow the formation of a stable soft coatings on implant material and increases their utility for various biomedical applications. In this chapter, we have discussed the conjugation strategies employed for the development of various dendrimer based implants coatings and their applications.

Till date, numerous conjugation strategies have been proposed by different research groups to fabricate various nanocomposite coated implant surface, specific for the desired application. The terminal functionalities of the dendrimer actually play the major role in the conjugation with the implant surface. To increase the stability of dendrimers on the implant material, certain modifications have been induced to enhance the conjugation strength and sustained activity. The pristine or engineered dendrimer including their nanocomposites can be conjugated on implant substrates by means of non-covalent (electrostatic interactions, or physical adsorption) or covalent interaction depending upon the specific application. Among these, the electrostatic interactions based conjugation strategies have been explored extensively, where the charged groups on the dendrimer or dendrimer based nanocomposites interact with oppositely charged group present on the implant surface. On the other hand, covalent conjugation and physical adsorption have been explored by a very few research groups.

Various properties of the dendrimer-coated implant materials make them a perfect candidate for applications such as: (1) remineralization of dentin (hydroxyapatite-rod-like/flake-like structures); (2) antibacterial agent, where they prevent the bacterial growth or biofilm formation on the implant material; (3) promote cell adhesion, and (4) bioactives delivery. The emphasis is given more on the functional coatings developed on bone or dental implants, and it can be concluded that the process of bone regeneration or osseointegration and HA remineralization can be significantly improved by encapsulating bone growth factors or other functional moiety in the dendrimer or by surface coating on the dendrimer. For example, use of insulin growth factor-1 as coating material over modified PAMAM dendrimers can aid in curing osteoarthritis (Geiger et al. 2018).

In relation to future prospective, some unexplored families of dendrimers suchas, peptide dendrimers, polyglycerol dendrimer, melamine dendrimer, and polyether-copolyester (PEPE) dendrimers, etc. can be explored to develop highly stable implant coatings. Further, the functional properties of the dendrimers can be enhanced via conjugation with other polymeric materials or bioactive molecules. Further, till date, the development of the implants coatings is mostly limited to

the laboratories, which causes the lack in the information regarding the clinical potential of the fabricated coating in real-time. To address this issue, clinical trials of the developed implant coatings is of utmost importance. In future, the focus on the translational studies in this field may result in creation of an economical product, with high success rate along with minimum or no side effects.

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Application of Nanotherapeutics for Combating Human Protozoan Parasitic Infections

7

Riti Mehta and Souvik Sengupta

Abstract

Human protozoan parasitic diseases are major issue of concern mainly in the tropical regions of the world. Human protozoan parasitic diseases that have high morbidity and mortality rate include malaria, leishmaniasis, and toxoplasmosis. The convetional treatments involved are not cost-effective and thus can't be afforded by everyone. Also, vaccinations against these diseases have achieved limited success due to the incredible smartness of these protozoan parasites. Due to this, chemotherapy remains the mainstay for treatment and often high drug doses are administered leading to severe side effects and drug resistance in the parasites. The application of nanotechnology seems to be an attractive alternative approach to the conventional method. Nanoparticles are more effective as they have higher bioavailability, increased clearance rate and they can be engineered to be target-specific wherein they can affect the diseased cells only. This chapter discusses the current treatments used against these protozoan parasites and also their shortcomings. Different types of nanoparticles have been designed to target the parasites such as lipid-based, metallic/inorganic and polymeric based nanoparticles. Since nanoparticles are less toxic and can be engineered to be more effective in controlling and preventing parasitic diseases, it can show the way for future anti-parasitic treatments using nanotherapeutics.

Keywords

Protozoan parasites · Malaria · Leishmaniasis · Toxoplasmosis · Immune system · Nanotechnology · Polymeric nanoparticles · Liposomes · Nanotherapeutics

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7.1 Introduction

Human protozoan parasitic diseases are a major issue of concern mainly in the tropical regions of the world. These parasites pose a significant threat to the human population, leading to poor health and adverse socio-economic situation worldwide (Andrews et al. 2014). Human protozoan parasitic diseases that have high morbidity and mortality rate mainly include malaria, leishmaniasis, and toxoplasmosis. These pathogens compromise the immunity of the host. Conventional treatments to these dreaded diseases involve boosting the immune system through medication and administering drugs and various vaccines. However, all of these have become less effective eventually. Firstly, the treatments involved are not cost-effective which can't be afforded by everyone. Secondly, vaccinations against these diseases have achieved limited success due to the incredible smartness of these protozoan parasites. Due to this, chemotherapy remains the mainstay for treatment and often high drug doses are administered leading to severe side effects and drug resistance in the parasites. Most of the drugs pose serious side effects which can be fatal. Hence, to overcome this condition, a new approach should be introduced.

One possible solution to combat the protozoan parasitic diseases is the use of nanomaterials. Various formulation of nanoparticles can be used to assure greater bioavailability, increased clearance rate, and they can be engineered to be target-specific. Moreover, they have other benefits over microparticles such as improved drug encapsulation, pharmacokinetics, and therapeutic potential. They include colloidal drug carriers such as liposomes, metallic nanoparticles and polymer based nanoparticles. Figure 7.1 schematically summarizes the structure of different type of nanoparticles. They provide protection against oxidoreduction and enzymatic reaction, increase bioavailability and reduce the effective dose (Arias et al. 2015). In general, nanomaterials can act either in a direct way on a microorganism or as carriers. In the later role, drugs or molecules can be directed to the specific target inside a particular intracellular pathogen.

The use of colloidal drug carriers like liposomes provide versatility in site-specific or targeted drug delivery along with controlled optimal drug release. Liposomes are largely used to target pathogens as it has improved solubility of lipophilic and amphiphilic drugs. Due to the presence of phospholipid bilayer, they are not recognized by the reticuloendothelial system (RES).

Nanoparticles can be dispersed in an aqueous phase with size ranging from 1 to 1000 nm in diameter. They may be composed of synthetic, semi-synthetic and natural polymers in which the active therapeutic molecule such as drug, antibody, peptides, oligodTs can be entrapped, dissolved, absorbed or adsorbed. Synthetic polymers include poly(D, L-lactic-co-glycolic acid) (PLGA), polyethylene glycol (PGE), or polyester bio beads. Natural polymers include proteins like gelatin, albumin, lectin, legumin, and vicillin and polysaccharides like alginate, dextran, pullulan, and chitosan. Due to the different types and forms, the versatility of nanomaterials provide advantages with potential for application in diagnosis, treatment, and immunizations against various infectious diseases, including toxoplasmosis (Assolini et al. 2017).

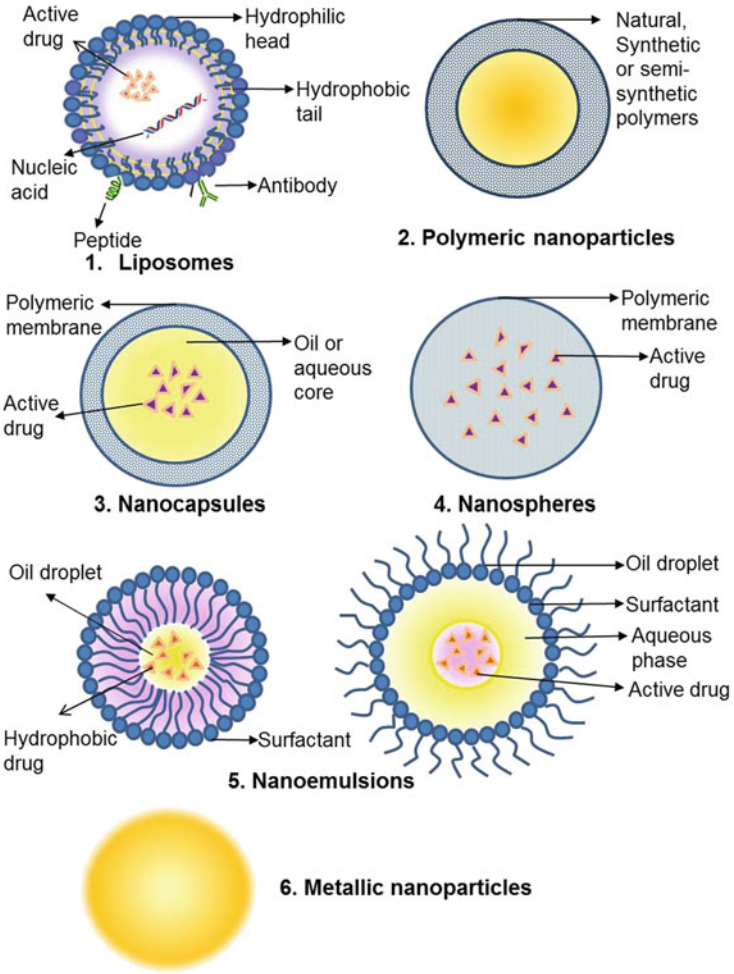

Fig. 7.1 Schematic representation of the structure of various nanoparticles

Nanocapsules are systems composed of vesicles in which the compound or molecule is confined in an aqueous or oily cavity coated by a membrane of the material used (Baldissera et al. 2017). In contrast, Nanospheres are systems which house the compound or molecule in a dispersed manner into particles in the matrix (Baldissera et al. 2017).

Metallic nanoparticles are based on well-defined small noble metal clusters in the state of zerovalent. Most common are the silver nanoparticles, which have antimicrobial and anti-inflammatory activity as well as utility in imaging. Silver nanoparticles can also form conjugates with antibodies, while iron nanoparticles have magnetic properties and potential in the management of tumorigenesis.

Nanoemulsions are different from metallic nanoparticles. Nanoemulsions are heterogeneous systems consisting of two immiscible phases, oil in water (O/W), in which the particle core is composed of water, and water in oil (W/O), in which the particle core is composed of oil. In addition, it is important to mention the solid lipid nanoparticles that have a lipid core in the solid-state. These two types of lipid nanomaterials allow the controlled release of drugs or molecules (Kimani et al. 2019).

The main strategies for targeting antimalarial drugs to the infected erythrocytes and occasionally to the hepatocytes using nanocarriers by the intravenous route are passive and active targeting. Passive targeting is achieved using conventional nanocarriers (e.g., liposomes, hydrophobic polymeric nanoparticles), or surface-modified long-circulating nanocarriers (e.g., PEGylated). In contrast, active targeting is attained by means of nanocarriers surface-modified with specific ligands such as carbohydrates, proteins, peptides or antibodies (Santos-Magalhães and Mosqueira 2010).

7.2 Malaria

Malaria has been documented as one of the oldest human diseases, which remains to be the most prevalent human infectious disease in the present era, caused by a protozoan parasite of the genus *Plasmodium*. Five different species of *Plasmodium* are responsible for causing this disease: *P. falciparum, P. vivax, P. malariae, P. ovale*, and *P. knowlesi* from which the first two are more virulent and prevalent in endemic regions (Santos-Magalhães and Mosqueira 2010). Certain cases of co-infection have also been reported. A survey conducted by WHO stated that in the year 2015, 214 million cases of malaria were reported with an estimated 438,000 thousand deaths in the year 2015 (Acharya et al. 2017). Malarial parasites are found in four stages: sporozoite that invades the liver hepatocytes, merozoites that infect erythrocytes, metabolically active trophozoites and schizont in which it replicates. Drugs that inhibit merozoite invasion are required but no drug is found to be effective (Santos-Magalhães and Mosqueira 2010; Geleta and Ketema 2016).

7.2.1 Organisms Causing Malaria

Infections specifically caused by *P. falciparum* can be fatal and results in three different conditions—asymptomatic malaria, mild malaria, and severe malaria. Asymptomatic malaria can occur in the presence of parasites in the peripheral circulation. However, it is not accompanied by any kind of normal symptoms of malarial infection such as chills and fever in the absence of any antimalarial treatment. Such conditions can be a result of partial immunity that regulates infection, although not eliminating it. Mild malaria is detected by parasitemia in peripheral blood smears along with fever and chill and acute symptoms, known as uncomplicated malaria. Severe or complicated malaria occurs with an increase in

the severity of malaria infection and possessing acute symptoms that affect the functioning of the brain in cerebral malaria along with several other organs (Acharya et al. 2017).

The malaria parasites *P. vivax* in particular, are believed to have perfected the art of benign virulence and survival within human beings. However, recent reports of severe malaria during *P. vivax* infections have given rise to several questions about *P. vivax* interactions with its human host (Acharya et al. 2017).

7.2.2 Current Drugs Available for Treatment

Over the years, many anti-malarial drugs have evolved in order to cure the disease. Table 7.1 enlists the current anti-malarial drugs used, their mode of action and side

Table 7.1 List of the current antimalarial drugs, mode of action and side effects

Drug	Mode of action	Drawback
Chloroquine (CQ)	Interferes with polymerization of toxic heme monomers released by digestion of haemoglobin (Slater 1993)	Parasite strains have developed CQ-resistance
Primaquine	Inhibits macromolecular biosynthesis (Olenick and Hahn 1972)	Very toxic drug
Quinine	Interferes with parasites ability to digest haemoglobin (Foley and Tilley 1997)	Vomiting, stomach cramps, diarrhea, nervousness, and confusion
Amodiaquine	Accumulates inside parasite food vacuole and interferes with heme detoxification	Risk of hepatotoxicity and agranulocytosis
Tetracycline	Target apicoplast or mitochondrion (Dahl et al. 2006)	Side effects include nausea, vomiting, diarrhea, and photosensitivity
Tafenoquine	Molecular target not known (Ebstie et al. 2016)	Causes acute hemolytic anemia with G6PD deficiency (Ebstie et al. 2016)
Mefloquine	Inhibits protein synthesis (Wong et al. 2017)	This leads to neuropsychiatric side effects and stillbirth in pregnancy
Halofantrine	Forms toxic complexes with ferritoporphyrin IX that damage the membrane of the parasite	Cardiac conduction abnormalities
Clindamycin	Target apicoplast (Lell and Kremsner 2002)	Slow onset of action
Pyronaridine	Inhibit glutathione-dependent degradation of hematin (Auparakkitanon et al. 2006)	Headache, vomiting, abdominal pain, bradycardia, and hypoglycemia
Malarone (combination of proguanil and atovaquone)	Inhibits electron transport to cytochrome bc1 complex (Fry and Pudney 1992)	Developed resistance

effects. Most of the antimalarial drugs target the erythrocytic stage of the infection, which is considered as the phase that causes symptomatic illness. Pretreatment of erythrocytes with drugs suggested that halofantrine, lumefantrine, piperaquine, amodiaquine, and mefloquine diffuse into and remain within the erythrocyte and inhibit downstream growth of parasites. All drugs inhibited parasite replication when added at ring stages, but only artesunate, artemisinin, cycloheximide, and trichostatin A appeared to have substantial activity against ring stages, whereas the other drugs acted later during intraerythrocytic development. When drugs were added to late schizonts, only artemisinin, cycloheximide, and trichostatin A were able to inhibit rupture and subsequent replication (Santos-Magalhães and Mosqueira 2010). Factors responsible for the emergence of resistance in different species of Plasmodium include (1) the rate of mutation in malarial parasites, (2) the costs associated with the resistance mutations, (3) the total load of the parasite, (4) the methods of drug selection and (5) the treatment compliance (Hastings 2004).

7.2.3 Use of Nanotechnology in Malaria

Due to genetic diversity, the parasites have evolved to survive harsh conditions and developed complex mechanisms of drug resistance. To overcome the limitations of antimalarial drugs, Nanotechnology offers greater opportunities to curb the disease by enabling tools for structural modification of chemical compounds that are more efficient and highly specific and selective. Various therapeutic approaches have been put forward for an easy and safe treatment of malaria. The use of nanocarriers as vehicles for the delivery of antimalarial drugs offer increased efficacy due to their site-specific action in less time, thereby reducing administration time between doses and promoting drug adherence. The different types of nanoparticles used for treating malaria are tabulated in Table 7.2.

Resistance to Chloroquine in *P. falciparum* occurs as a result of mutations on *P. falciparum* chloroquine transporter gene (pfcrt) and *P. falciparum* multi-drug resistance gene (pfmdr1) respectively. The protein transporters exhibited as products of the above gene expression are CQ resistance transporter (CRT) and P-glycoprotein homolog 1 (Pgh 1). Both transporters are present on the digestive vacuole membrane of *Plasmodium* and CQ-resistant *Plasmodium* strains accumulate a lesser amount of drug in the digestive vacuole and prevent themselves from drug damage. A new strategy developed to bypass CQ-transporters is the formation of drug encapsulated nanosystems that are able to pass the digestive vacuole membrane either through membrane fusion or membrane-induced instability triggered by pH difference. An effective nanosystem can be designed that encapsulates CQ in nanocarriers. One such formulation has been reported in which CQ is entrapped in pH-sensitive liposomes (Santos-Magalhães and Mosqueira 2010).

The efficacy of silver in counteracting bacteria seems ascribable to the action of Ag ions, which are toxic to microbes but apparently almost innocuous to humans, for we can tolerate relatively high levels of this metal without any adverse effect, probably through the deposition of excess silver as insoluble silver chloride or

Table 7.2 Types of nanoparticles used against malarial parasites

Type of nanoparticle	Drug used	Effect
Nanocapsules (NC)	Halofantrine (Santos-Magalhães and Mosqueira 2010)	Delays phagocytosis Increases half-life of the drug (Santos-Magalhães and Mosqueira 2010)
Liposomes	Curcuminoids-loaded liposomes + α/β arteether (Aditya et al. 2012)	Targets schizont stage of falciparum (Aditya et al. 2013)
	Curcuminoids-loaded liposomes + artemisinin (Aditya et al. 2013)	
Immunoliposomes	DOPC and CHOL as surface material + chloroquine and fosmidomycin as the core drug (Urbán et al. 2011)	Tenfold increase in antimalarial activity (Urbán et al. 2011)
Gel state liposomes	Chloroquine	Increased bioavailability (Santos-Magalhães and Mosqueira 2010)
Neutral liposomes	Quinine (large unilamellar vesicles) (Santos-Magalhães and Mosqueira 2010)	Increased uptake of the drug (Santos-Magalhães and Mosqueira 2010)
	Chloroquinine (large unilamiller vesicles)	Increased uptake of the drug (Santos-Magalhães and Mosqueira 2010)
	Artemether (multilamellar liposomes) (Santos-Magalhães and Mosqueira 2010)	100% encapsulation efficiency (Santos-Magalhães and Mosqueira 2010)
	Artesurate (Santos-Magalhães and Mosqueira 2010)	100% encapsulation efficiency (Santos-Magalhães and Mosqueira 2010)
Nucleic acid based NPs	microRNAs + chitosan particles (Rahman et al. 2019)	Target topoisomerase II gene (Rahman et al. 2019)

sulfide, or metal aggregates. Silver-based antiplasmodial compounds prepared by a series of mono and dinuclear silver and gold complexes containing mono- and bis (N-heterocyclic carbene) (NHC))-based ligands, all N-functionalized with different groups (amide, alcohol, and nitrogen-containing heterocycles such as quinoline and bipyridine) were tested on a chloroquine-resistant strain of *P. falciparum*. They interact strongly with the thiol and phosphate groups present in enzymes and in bacterial DNA and proteins which stops the bacterial cell from performing vital functions such as respiration and replication leading to cell death (Rai et al. 2017).

7.2.4 Red Blood Cell-Mediated Therapy

Plasmodium resides in parasitophorous vacuole formed in the host RBC and this provides an appropriate site to promote high antimalarial drug concentration. These

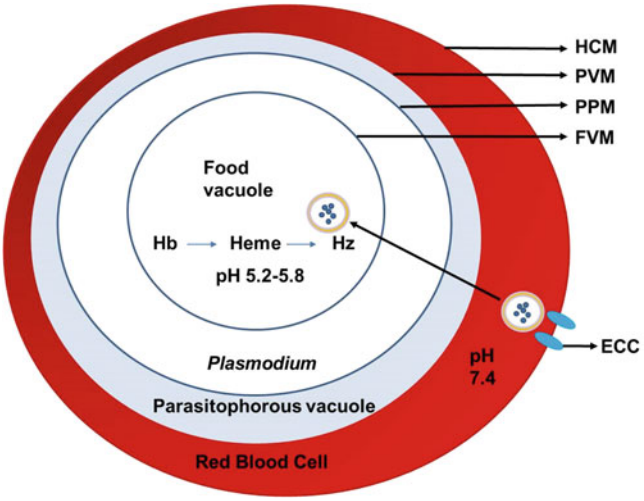

Fig. 7.2 Schematic representation of the action of nanocarriers (liposomes, metallic nanoparticles, nanoemulsions) towards parasite residing in RBC. The drug interacts with Hemazoin (Hz)

parasites are not easily accesible and the drugs should penetrate through multiple barriers to target *plasmodium* and show its activity (Fig. 7.2). The barriers begin from the RBC membrane to the desired organelle membrane in plasmodium—the host cell membrane (HCM), the parasitophorous vacuolar membrane (PVM), the parasite plasma membrane (PPM) and site-specific food vacuole membrane (FVM) or an endoplasmic reticulum membrane (ERM) (Santos-Magalhães and Mosqueira 2010).

Under normal circumstances, phospholipid metabolism is absent in mature RBCs but in infected cells, it increases by 50% due to the de novo biosynthesis pathway of phosphatidylcholine (PC). Choline is transported from serum to RBC which is controlled by Erythrocyte choline carriers (ECC). The parasite-encoded ECC involved in choline uptake by infected-RBCs offers great potential for the selective targeting of potent antimalarial drugs. These drugs specifically accumulate inside infected RBCs, block phosphatidylcholine de novo biosynthesis and interact with hemozoin (Hz) (Gnanadesigan et al. 2019).

Moreover, it is reported that the membrane of infected RBC tends to have greater permeability to a large number of lower-molecular weight solutes post-infection by Plasmodium. Infected RBCs can be differentiated from non-infected ones through a membrane channel known as the 'New permeability pathway' (NPP) that opens after 12–16 h of infection. NPPs can be considered as a new approach to incorporate nanocarriers of <80 nm diameter to affect intracellular compartments of parasite (Santos-Magalhães and Mosqueira 2010).

7.2.5 Passive Drug Targeting

Cells of the mononuclear phagocyte system (MPS) are considered as the best drug target due to their phagocytic activity. Taking this into consideration, nanocarriers can be used for passive targeting, depending on the pathophysiological and anatomical features of host cells. On the contrary, RBCs are deprived of phagocytic and endocytic activity and this makes delivering drugs difficult. However, in the case of malaria where the target cell is RBC, if the drug is engulfed by MPS cells, the drug will be delivered in macrophages instead of RBCs. Thus, the overload of nanocarriers in exposed phagocytes could lead to an initial blockage of the phagocytic uptake that is resolved within 24–48 h. Due to the rise in macrophage capacity by 2–3 times, the activity of drugs entrapped in nanocarriers could diminish. On the other hand, it could be beneficial for the pharmacokinetic profile of a drug as the drug would release into the blood (Scherphof et al. 1997).

Surface Modified Nanocarriers

Surface-modification is performed on nanocarriers with the help of hydrophilic polymers such as polyethylene glycol (PEG) that delays phagocytosis. The drug is accompanied by prolonging half-life in the blood, leading to modulation of the pharmacokinetic profile of the drug. Halofantrine (Hf)-loaded-nanocapsules (NC) is one such example (Santos-Magalhães and Mosqueira 2010). Halofantrine, a very hydrophobic drug used in the treatment of malaria was formulated in two ways for intravenous administration: (1) poly-lactic acid (PLA)-NC without surface modification and (2) PLA modified with PEG (PLA-PEG) NC. However, when tested on *P. berghei*-infected mice, there was a negligible difference between the pharmacokinetic parameters of Hf encapsulated in both formulations despite the biodistribution profiles of unloaded NC being different.

7.2.6 Active Drug Targeting with Surface-Modified Nanocarriers

Active targeting can be achieved by conjugating a cell-specific ligand at the surface of the carrier. This approach may be successful if the receptors for surface-bound ligands are expressed uniquely in diseased cells or if their expression is differentially higher in diseased cells as compared to normal ones. The disadvantage in this approach is that ligand-attached nanocarriers may induce an undesirable immunological response, due to the proteic nature of some ligands. Another challenge is to adjust the number of ligands per nanocarrier and the suitable PEG chain length at the surface of nanocarriers to properly expose the ligand for cell recognition. Thus, the targeting of moieties that are natural candidates for attachment to nanocarriers are peptides, antibodies and, particularly, small carbohydrate-based molecules (Santos-Magalhães and Mosqueira 2010).

Liposomes as Nanocarriers for Antimalarials

Liposomes are vesicles consisting of one or several concentric spheres of phospholipid bilayers with an aqueous cavity in the center. This aqueous core can be incorporated with hydrophilic drugs or active compounds enclosed by lipophilic drugs imbibed into the phospholipid bilayer. To develop a target-specific system, the surface of liposomes are modified with antibodies, peptides or other ligands (Santos-Magalhães and Mosqueira 2010). The conventional liposomes to encapsulate antimalarials were long-circulating negatively-charged liposomes prepared with EPC, CHOL and phosphatidylglycerol (PG) or phosphatidylethanolamine (PE) conjugated to PEG (Qiu et al. 2008).

Economically viable soy phosphatidylcholine (soy PC) was used to formulate curcuminoids-loaded liposomes. The antimalarial activity was evaluated on *P. berghei* infected mice through administering curcuminoids-loaded liposomes alone and in combination with α/β arteether which is an ethyl ether derivative of artemisinin effective against the schizont stage of *P. falciparum*. There was a higher survival rate with lower parasitemia in mice treated with curcuminoids-loaded liposomes when compared to the control group (no treatment and free curcuminoids treated group). Importantly, the combination therapy of curcuminoids-loaded liposomes along with α/β arteether was able to not only cure infected mice but also prevented recrudescence (Aditya et al. 2012).

Another formulation was made in which artemisinin (50 mg/kg) and curcumin (100 mg/kg) alone or in combination were loaded in conventional and PEGylated liposomes and fabricated by film hydration method using cholesterol (CHOL), P90G and Poly (ethylene glycol)-2000-di stearoyl phosphatidylethanolamine. For intraperitoneal administration, the size of conventional and PEGylated liposomes was maintained $\leq$200 nm with the encapsulation efficiency ~70% and zeta-potential of around −20 mV. The treatment on *P. berghei* NK-65 infected murine malaria model reduced the parasitemia more rapidly compared to the free drug solution at the same drug concentration (Aditya et al. 2013).

Immunoliposomes using 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and CHOL as surface material and chloroquine and fosmidomycin as core material was designed with pRBC-specific monoclonal antibody BM1234. These antibodies are specific to RBC in the late maturation stage of *Plasmodium*. *In vitro* experiments depicted tenfold increases in antimalarial activity when these antimalarial drugs were encapsulated in immunoliposomes compared to free drugs and/or after encapsulation, drug concentration required to eliminate a specific number of parasites was ten times less compared to unencapsulated drugs. In addition, due to the presence of BM1234 antigen on trophozoites and schizonts stages compared to the ring stage, trophozoites and schizonts synchronized parasite culture showed more parasitemia reduction (Urbán et al. 2011).

Gel state liposomes with chloroquine as the core material were prepared by using distearoyl phosphatidylcholine (DSPC), CHOL and dipalmitoyl phosphatidylglycerol (DPPG) as lipid components bearing negative charge. These liposomes were tested for their therapeutic efficiency on *P. berghei* infected mice revealed enhanced therapeutic efficiency when chloroquine was encapsulated in

liposomes. Pharmacokinetic studies carried out with the same formulations when compared to free chloroquine and chloroquine-loaded liposomes revealed a prolonged availability of the drug in whole blood, plasma and RBCs after intraperitoneal administration (Aditya et al. 2013).

Nucleic Acid-Based Nano-Therapy of Malaria

Some microRNAs have played a satisfactory role in preventing malaria. An effort has been made which showed that *P. falciparum* has a susceptibility to antisense oligonucleotide NPs (ODN-NS). This fascinating method generally utilizes antisense oligo deoxy (OD) N-chitosan particles which are 50 nm in size. These particles increase the antisense ODN internalization by *P. falciparum*-infected erythrocytes through erythrocyte permeation pathways that target the *Plasmodium* topoisomerase II gene (Rahman et al. 2019).

Polymeric Nanoparticles as Nanocarriers for Antimalarials

The surface properties of the Nanospheres (NS) can be modulated using a wide variety of polymers in order to obtain passive or active targeting in the body (Santos-Magalhães and Mosqueira 2010).

Dendrimers are homogenous and monodispersed, radially symmetric molecules with a dimension in nanometers consisting of tree-like branchers (Abbasi et al. 2014). Poly-L-lysine dendrimers coated and uncoated with chondroitin sulfate A (CSA) was used for sustained delivery of chloroquine phosphate (CQ) as potential blood schizonticide. Study on albino rats indicated that there was prolonged and controlled release of CQ after intravenous administration (Bhadra et al. 2006).

7.2.7 Vaccines Developed for Malaria

Vaccines generate an immune-based response to protect people from infection and stop transmission of the foreign agents. In order to induce a successful immune response, uptake of antigen by antigen-presenting cells is essential. Hence, apart from a drug delivery system, nanoparticles also play a role in carrying antigens and work as effective vaccines (Kumar et al. 2015b). These vaccines target various lifecycle stages of the parasite in the liver, blood or sexual stage in the mosquito (Doumbo et al. 2018).

Plasmodium falciparum consists of Pfs230, Pfs48/45 and Pfs25 proteins which are the primary target of Transmission—blocking vaccines (TBV), acting upon the sexual stage of the parasite. Pfs25 is expressed on the surface of zygote and ookinetes, which are promising targets of TBV. Codon-harmonized recombinant Pfs25 (CHrPfs25) was expressed in *E. coli* and was purified after oxidative refolding that retained reduction sensitive conformational epitopes of transmission blocking monoclonal antibodies (Kumar et al. 2015b). Oxidative refolding is a process of formation of disulphide bonds between cysteine residues through redox reaction. This successful refolding, purification and monomeric conformation of CHrPfs25 proved to be a transmission-blocking antibody in mice. Gold nanoparticles are used

for the delivery of vaccines due to their small size, shape, biocompatibility and easy surface modifications. Further, the efficacy of AuNPs was also determined by co-administering it with conventional adjuvant alum (Kumar et al. 2015b).

To eradicate *P. vivax* infection, recombinant antigen—vivax malaria protein (VMP001) was developed from circumsporozoite protein (CSP) which is most prevalent on sporozoite membrane. Poly-lactic-coglycolic acid (PLGA) activate the inflammasome in APC via NLRP3 and enhance adaptive response. PLGA core can be modified with pathogen-associated molecular patterns (PAMPs) and protein antigens. Through this strategy, monophosphoryl lipid A (MPLA) was incorporated into lipid membranes, creating pathogen mimicking nanoparticle vaccines (VMP001-NPs). Antibody response was generated by mixing conventional adjuvant like Montanide with VMP001 (Moon et al. 2012).

7.3 Leishmaniasis

Leishmaniasis is a tropical disease caused by *Leishmania* protozoa that are transmitted to mammalian hosts by sandfly. It causes human disease with a wide range of severity ranging from self-healing cutaneous lesions to fatal visceral infections. Human macrophages are hosts for these parasites and natural hosts include rodents, small mammals, and dogs. According to the 2014s report, every year one to two million people are diagnosed with this deadly disease. These parasites are found in 88 countries around the world. However, over 90% of potentially fatal infections occur in just six countries: Brazil, Ethiopia, Sudan, South Sudan, India, and Bangladesh (Roberts et al. 2000). Leishmaniasis disseminates through the lymphatic and vascular systems and infects monocytes and macrophages in liver, spleen, bone marrow and lymph nodes (Akbari et al. 2017).

Leishmaniasis is a neglected tropical disease and the Leishmania parasites have a dimorphic life cycle in which the parasites reside as extracellular promastigotes in the vector and as intracellular amastigotes in mammalian host macrophages (Roberts et al. 2000).

Once the parasite has gained access to the mammalian host through a sandfly bite, it is taken up via receptor-mediated phagocytosis by resident skin dendritic cells or macrophages. In an elegant example of molecular mimicry, the organism uses the host complement receptors to gain access to the hostile environment of the phagolysosome, where, despite a pH of 4.5–5.0 and activated proteinases, it thrives. Activated macrophages and T lymphocytes are recruited to the site of infection (Santos et al. 2008).

Visceral Leishmaniasis Symptoms include fever, weight loss, hepatosplenomegaly, and anemia. It can be fatal despite being treated. WHO has reported more than 95% cases of Visceral Leishmaniasis (2017) in ten countries: Bangladesh, Brazil, China, Ethiopia, India, Kenya, Nepal, Somalia, South Sudan and Sudan (Roberts et al. 2000; Santos et al. 2008).

Cutaneous Leishmaniasis In this form of Leishmaniasis, there is the formation of a skin ulcer at the spot of the sandfly bite. It might heal within months leaving a scar. Certain rare forms may cause skin lesions. Ninety-five percent case occur in America, Mediterranean basin, Middle East and Central Asia. (Roberts et al. 2000; Santos et al. 2008).

Mucocutaneous Leishmaniasis This is an uncommon consequence of cutaneous leishmaniasis that may be present even years after the initial skin ulcer has healed. This metastatic complication of the primary lesion results in disfiguring and progressive ulceration at the nasal mucocutaneous junction. Occurrence is mainly in Brazil, Ethiopia, and Peru (Santos et al. 2008), (Roberts et al. 2000).

7.3.1 Current Drugs Available for Treatment

The treatment options for leishmaniasis are limited including pentavalent antimonials, pentamidine, Amphotericin B (AmB), liposomal formulation of Amphotericin B and miltefosine, which have been introduced recently in the group of antileishmanial drugs. The current drugs available for treating Leishmaniasis with their mode of action and side effects are tabulated in Table 7.3.

7.3.2 Use of Nanotechnology in Leishmaniasis

Using nanoparticles as a tool to cure leishmaniasis, several approaches have been considered. First is the design of nano-drug delivery systems for common and conventional drugs. Such a drug delivery system improves the efficacy and decreases the toxicity with effect on the metabolism of drugs including absorption, distribution and excretion. Thus, nano-drug delivery systems result in high efficacy, high target delivery effect, low toxicity, high concentration, releasing drugs with controlled system and prolonged systemic circulation lifetime. The second approach

Table 7.3 Current antileishmanial drugs, their mode of action and side effects

Drug	Mode of action	Drawback
Amphotericin B	Binds with ergosterol forming pores in the cell membrane (Mesa-Arango et al. 2012)	High toxicity to host cells (Mesa-Arango et al. 2012)
Miltefosine	Interacts with lipids, inhibits cytochrome c oxidase leading to apoptosis-like death (Pinto-Martinez et al. 2018)	Teratogenic and long half-life (Dorlo et al. 2012)
Pentavalent antimonials	Inhibits adenosine diphosphate phosphorylation of DNA I topoisomerase (Chakraborty and Majumder 1988)	Drug resistance up to 65%
Pentamidine	Inhibition of synthesis of DNA, RNA, phospholipids and proteins (Bornstein and Yarbro 1970)	Hypotension, hypoglycaemia and nephrotoxicity (Babokhov et al. 2013)

Table 7.4 Types of nanoparticles used against Leishmania parasites

Type of nanoparticle	Drug used	Effect
Polymeric nanoparticles	Primaquine in poly-alkyl cyanoacrylate (PACA) nanoparticles (Akbari et al. 2017)	Twenty one times more effective in eradicating parasites (Akbari et al. 2017)
	Desoxycholate amphotericin B loaded PLGA nanoparticles (Silveira et al. 2009)	Effectively reduce parasite cell viability (Silveira et al. 2009)
	Sitamaquine encapsulated in PLGA-PEG (Kumar et al. 2015a)	Three times more effective than the free form of sitamaquine (Kumar et al. 2015a)
Metal oxide nanoparticles	Silver doped titanium dioxide nanoparticles (TiAg NPs) (Allahverdiyev et al. 2013)	ROS generated impairs the metabolic activity (Allahverdiyev et al. 2013)
	Selenium nanoparticles (Akbari et al. 2017)	Inhibits proliferation of amastigote and promastigote form (Akbari et al. 2017)
	Rhenium (V) and gold (III) (Akbari et al. 2017)	Inhibits the activity of cysteine protease (Akbari et al. 2017)
Liposomes	AmBisome® (de Souza et al. 2018)	Aqueous pores formed within the plasma membrane of parasites Increases permeability of monovalent cations and metabolites Binds to ergosterol present on the cell membrane (de Souza et al. 2018)
	Paromomycin (de Souza et al. 2018)	Low cytotoxicity and increased efficacy (de Souza et al. 2018)
	Trifluralin (Carvalheiro et al. 2015)	Effective against visceral leishmaniasis Targets amastigote form of *L. infantum* (Carvalheiro et al. 2015)
Nanoemulsions	Chalcone (de Souza et al. 2018)	Effective against cutaneous leishmaniasis Targets amastigote form of L. amazonensis
Solid lipid nanoparticle	AmB and Miltefosine (de Souza et al. 2018)	Effective against visceral leishmaniasis (de Souza et al. 2018)

to drug formulation is the nanotization of the drug. Macrophages phagocytose the nanoparticles as foreign bodies causing to target specific delivery for Leishmania inside macrophages (Akbari et al. 2017). The different types of nanoparticles used for the treatment of Leishmaniasis are shown in Table 7.4.

Polymeric Nanoparticles

For long circulation time and quick eradication from the body by phagocyte system, polymeric nanoparticles such as nanocapsules and nanospheres have proved to be a

strong treatment. These particles introduced through the passive drug delivery system as the mechanism of action is enhanced with a reduced requirement of dose.

Nanocapsules formed by loading primaquine in poly-alkyl cyanoacrylate (PACA) nanoparticles proves to be effective against *Leishmania donovani* in macrophages. Compared to the free form of primaquine, the new formulation of the antileishmanial agent is 21 times more effective in eradicating parasites from the macrophage. Along with it nanoencapsulation tend to improve the bioavailability of drugs (Akbari et al. 2017).

Among the polymeric nanoparticle delivery systems, various forms of poly lactic-co-glycolic acid (PLGA) are formulated as active and passive carriers of antileishmanial drugs. The effect of desoxycholate amphotericin B loaded PLGA nanoparticles and dimercaptosuccinic acid (DMSA) was evaluated for the treatment of cutaneous leishmaniasis. This nano-drug appeared significantly more effective compared to free amphotericin B in terms of reduction of parasite number and cell viability (de Carvalho et al. 2013). Moreover, reduced dose frequency and increased dosing interval gave similar results as using free amphotericin B. Other PLGA formulations include saponin β-aescin loaded nanoparticles (Van de Ven et al. 2011).

Polymeric nanoparticles were made more specific against Leishmania infected macrophages by developing sitamaquine encapsulated in PLGA-PEG nanoparticles which were attached with an antibody against CD14 present on affected macrophages. These proved to be three times more effective than the free form of sitamaquine on hamsters treated with PLGA-PEG encapsulated form (Kumar et al. 2015a).

Natural drugs that show poor bioavailability and stability can be encapsulated in PLGA nanoparticles to improve its efficacy and reduce toxicity. Similarly, potential antileishmanial drug artemisinin loaded in PLGA reduced the viability of amastigotes and have been reported to be non-toxic to macrophages compared to free artemisinin (Akbari et al. 2017).

Metal Oxide Nanoparticles

Metallic oxide nanoparticles possess larger surface area due to which they have greater chemical reactivity and are capable to produce reactive oxygen species that kill parasites. Their minute size and large surface areas make it possible to interact with DNA and enzymes and disrupt vital activity and structures of the infectious agents. As an example, metallic compounds and metal oxide nanoparticles are effective in inhibiting the enzyme of trypanothione metabolism that is vital in the survival of Leishmania parasites.

Silver doped titanium dioxide nanoparticles (TiAg NPs) possess antileishmanial activity against *Leishmania tropica* and *Leishmania infantum*. These parasites are sensitive to reactive oxygen species as nanoparticles impair their biological functions including metabolic activity, viability and survival within the host macrophages. Although TiAg nanoparticles used alone in treatment are effective against visceral leishmaniasis, they can also be used in combination with visible light for the treatment of cutaneous leishmaniasis (Allahverdiyev et al. 2013).

Biogenic selenium nanoparticles can be used as a novel therapeutic agent in the treatment of the local lesions of cutaneous leishmaniasis. The nanoparticles of selenium inhibit the proliferation of amastigote and promastigote forms of *Leishmania major* in animal models *in vitro*.

Apart from these metallic nanoparticles, other metal compounds have also been tested for antileishmanial activity (Akbari et al. 2017):

- Zinc sulfate has more than 96% cure rates when it is used orally against cutaneous leishmaniasis.
- Platinum (II) salts completely prevent the growth of *L. donovani* amastigotes.
- Rhenium (V) and gold (III) have reported significant antileishmanial efficacies as they inhibit the activity of leishmanial enzymes especially cysteine protease.

Liposomes

One of the most common methods to prepare liposomes is through the thin-film hydration procedure (Fig. 7.3). First, the lipids are dissolved in organic solvents like trichloromethane and chloroform. Once the solvent evaporates, dry lipid film is formed. Then the film is hydrated with an aqueous solution, such as a buffer with pH 7–7.4 at a temperature higher than lipid transition temperature (Akbari et al. 2017). Drugs soluble in triglycerides (>50 mg/mL) and octanol: water partition coefficient (log P > 5) improve its absorption to the lymphatic system. So, triglycerides with longer chain are better transported to the lymphatic system than medium-chain (de Souza et al. 2018).

The best clinical treatment available in the market for curing visceral leishmaniasis is AmBisome®, which is a liposomal formulation of amphotericin B. L-AmB constitutes high-temperature phospholipids and cholesterol with AmB incorporated in the center of the bilayer. AmB is thought to form aqueous pores within the plasma membrane of parasites and increase the permeability of monovalent cations and metabolites. This action takes place after AmB is released from the liposome and it passes through the cell wall and binds to ergosterol present on the *Leishmania* cell membrane. However, L-AmB has a major drawback as it has a slow elimination rate from the body leading to nephrotoxicity, and also it has a high cost.

Paromomycin (PRM) antibiotic-loaded liposomes are used as an alternative to AmBisome®, targeting the spleen, lungs and liver which results in increased therapeutic efficacy and lower cytotoxicity in *Leishmania infantum* infected murine models.

Halofantrine is a drug approved by FDA for the treatment of malaria. However, Halofanrine is hydrophobic and poorly absorbed by the body. It shows cardiotoxic effects which can cause sudden death. A novel liposomal formulation of halofantrine has recently been shown to have potent anti-leishmanial effect (Bhagat et al. 2019).

The herbicide named trifluralin (TFL) was used to enhance antileishmanial activity by developing liposomal formulations TFL-A3 and TFL-A6. Its efficacy was tested *in vitro* against *Leishmania infantum* and *in vivo* against visceral leishmaniasis murine model, resulting in an IC_{50} of 1.2 ± 0.4 μM with TFL-A3 1.8 ± 1.3 μM with TFL-A6 respectively. It was further reported that the formulation

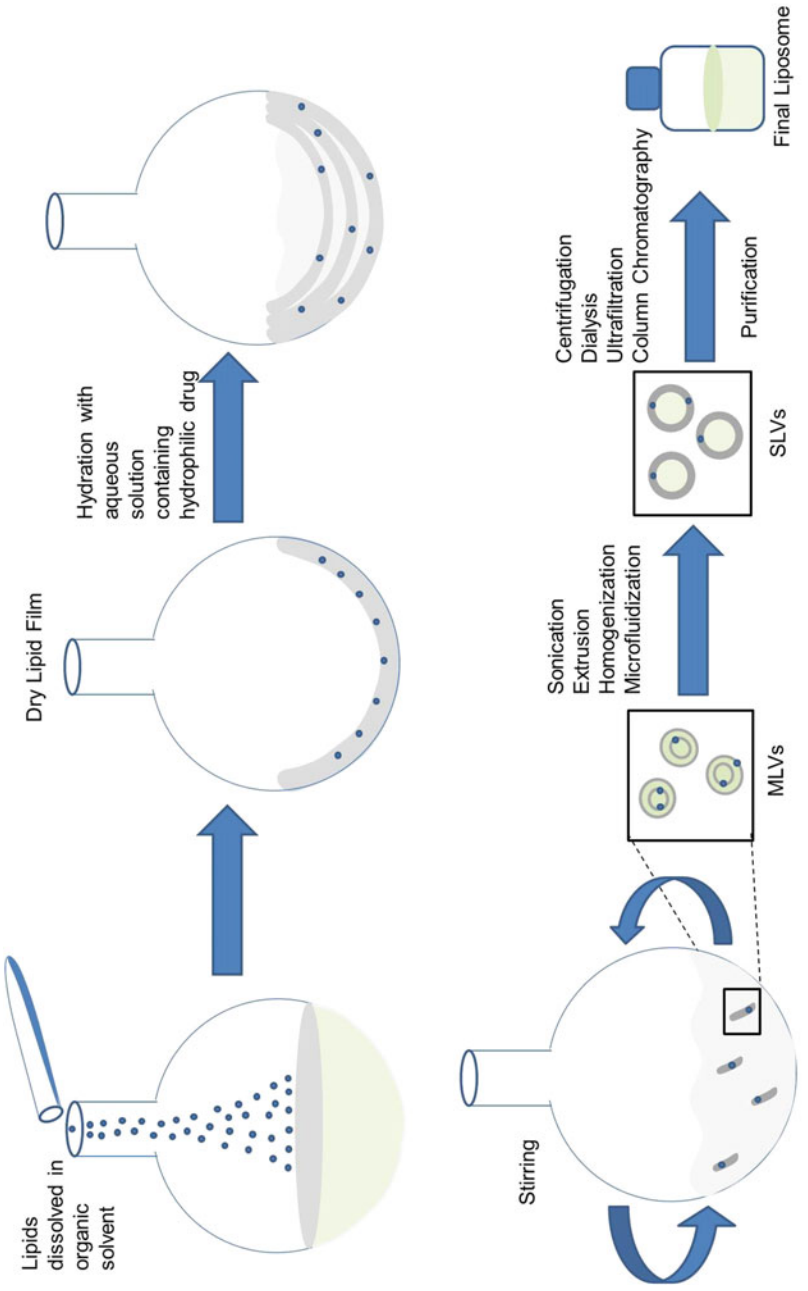

Fig. 7.3 Schematic representation of the formation of liposomes

was capable of targeting the amastigote form of *Leishmania infantum* residing in the host macrophages, proving to be an effective therapeutic drug (Carvalheiro et al. 2015).

Nanoemulsions and Solid Liquid Nanoparticles

Nanoemulsions (NE), a form of lipid colloidal particle, lie in the range of 10–1000 nm with lipid liquid, surfactant and aqueous phase as main components. These components form a dispersion of two immiscible phases and depending on the surfactant type they can be presented in oil-in-water or water-in-oil emulsion. They can even generate cationic or anionic nanoemulsions (de Souza et al. 2018).

Solid Lipid Nanoparticles (SLN) have replaced liquid lipids from emulsions to solid lipids, which result in particles of diameter 40–1000 nm and remain solid at room temperature. Further, these particles can be modified to form colloidal particles in which a matrix composed of a binary mixture of solid and liquid lipids is present in the core. These are known as Nanostructured lipid carriers (NLC).

NE, SLN, and NLC can be obtained through two main processes: mechanical force and physicochemical energy. The first process comprises of high-pressure homogenization, ultrasound and microfluidization. The particles are broken through shearing, collision and cavitational force. Whereas, in the second process the break down occurs through spontaneous curvature change of phases during the phase transition process.

NE was prepared by incorporating chalcone in it for treating cutaneous leishmaniasis. They were effective against amastigotes of *L. amazonensis* infected monocyte cell line (THP-1).

SLN were prepared using solvent evaporation and spontaneous emulsification method that entrapped poor water-soluble drug, like AmB with high entrapment efficiency of 76.5 ± 5%. SLN showed less toxicity towards normal kidney cells (293 T cells) compared to commercial AmB products, Fungizone® and AmBisome®.

Later, SLN based on nano-chelates was proposed. The stable cationic phospholipid precipitates comprise of positively charged calcium ions and negatively charged phospholipids combining two drugs AmB and miltefosine which is administered orally. The encapsulation efficiency was 54% and 59% for AmB and miltefosine and proved to be effective against visceral leishmaniasis (de Souza et al. 2018).

7.3.3 Vaccines Developed for Leishmaniasis

Leishmania expresses large quantities of cysteine proteinases (CPs) that are the major virulence factor and play an important role in causing infection. *L. major* consists of two important CPs: CPB (type I) present at amastigote developmental stage, CPA (type II) present at higher amastigote stage and secondary promastigote stage, and CPC (type III) present at all life cycle stages. Vaccines in a combination with CPA/CPB are used against visceral and cutaneous leishmaniasis but induce a

poor immunogenic response. Cationic Solid-lipid nanoparticles were used as delivery agents to overcome shelf-life stability, cost, and toxicity. Hence, non-viral DNA vaccine was formulated by producing immunogenic CPA and CPB genes with CSLNs consisting of cetyl palmitate, cholesterol, DOTAP and Tween 80 (Doroud et al. 2011).

7.4 Trypanosomiasis

African trypanosomiasis (AT), also known as sleeping sickness is caused by the bite of Glossina commonly known as Tsetse fly which transmits *Trypanosoma brucei* to animals and humans. The main parasites causing this African disease are *Trypanosoma brucei* and *Trypanosoma cruzi* (Adeyemi and Whiteley 2014). They reside in the bloodstream and lymph nodes after which they progress towards the central nervous system. *T. b. rhodesiense* and *T. b. gambiense* is responsible for causing trypanosomiasis in humans. Whereas, *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma evansi* cause trypanosomiasis in animals. African trypanosomiasis is endemic in African countries with 60 million people at risk creating a devastating impact on sub-Saharan Africa. These parasites are able to evade the host immune system by altering the Variant Surface Glycoprotein (VSG) by antigenic variation process (Kroubi et al. 2010; Arias et al. 2015).

The life cycle is comprised of three forms: (1) infective trypomastigotes found in mammalian blood and hindgut of the tsetse fly, (2) epimastigotes that proliferate, (3) amastigotes that multiply by binary fission in the host cell.

7.4.1 Current Drugs Available for Treatment

As shown in Table 7.5, pentamidine and suramin are suitable for treating first-stage *T. b. gambiense* Human African Trypanosomiasis (HAT) and *T. b. rhodesiense* Human African trypanosomiasis (HAT). Eflornithine, nifurtimox or melarsoprol is the available drug for controlling the advanced disease.

7.4.2 Use of Nanotechnology in Trypanosomiasis

Lipid Nanoparticles

4,4-(diazoamino) dibenzamidine (DMZ) is an anti-trypanosomal drug that binds to parasitic kinetoplast DNA via hydrogen bonds. The free drug is unable to cross the blood-brain barrier, causing toxicity to non-targeted cells and parasite resistance. To overcome this, DMZ was loaded in porous cationic nanoparticle with lipid core ($_{70}$DGNP^{+} nanoparticles). They are endocytosed by blood-brain barrier endothelial cells without activating the complement system (Kroubi et al. 2010).

Table 7.5 Current anti-trypanosomal drugs, their mode of action and side effects (Babokhov et al. 2013)

Drug	Mode of action	Drawback
Pentamidine (pentamidine Isothionate)	Disrupts mitochondrial membrane	Ineffective against stage II *T. b. gambiense* and both stages of *T. b. rhodesiense*
Suramin	Disrupts glycolysis by binding to glycosome	Ineffective against stage II *T. b. gambiense* and *T. b. rhodesiense*
Eflornithine (difluoro methyl ornithine)	Disrupts redox metabolism and glycolysis	Toxic, encephalopathy post-treatment, 30% resistance
Miltefosine	Disrupts proliferation and sensitive to oxidative attack	Ineffective against both stages of *T. b. rhodesiense*
NECT (nifurtimox–eflornithine combination treatment)	Oxidative attack on weakened parasites	Resistance to treatment

Polymeric Nanoparticles

Nanobodies are single-domain camelid functional heavy-chain immunoglobulins derived from camelids that are mammals of camelidae family that include such as lambs, camels and alpacas. Conventional antibodies have two variable domains (VH and VL) which offer stability to each other and binding specificity. Whereas, nanobodies lack VL domains and have HVV domains that possess high stability and hydrophilic side (Bever et al. 2016). Advantages of nanobodies over conventional antibodies are: (1) small size (15 kDa) (2) high affinity and specificity (3) high stability and solubility (4) economically viable and (5) can be easily produced in animals and microorganism.

Drug encapsulated polyethylene glycol (PEG) molecules increase nanoparticle circulation time by reducing liver uptake. There was a threefold reduction in half inhibitory concentration of pentamidine-PLGA nanoparticles compared to free drug. Further, targeting it with nanobody showed a fourfold reduction of parasite compared to passive targeting (Arias et al. 2015).

Quinapyramine sulfate (QS), an aminoquinaldine derivative is reported to be an effective drug against *T. evansi*. However, it also causes local and systemic reactions like salvation, trembling and, diarrhea. It even causes side effects on infected cattle, horses, dogs, and pigs. Hence, QS was formulated using an anionic polysaccharide agent sodium alginate which is biocompatible and nontoxic. The solubilization and sustained drug release are governed by a simple mechanism of sodium-calcium exchange in physiological fluids (Dahl et al. 2006). When calcium alginate enters an environment rich in monovalent salts, insoluble calcium is converted to soluble alginate making it soluble. They were able to clear the parasite at 4 h (Manuja et al. 2014).

Poly-ε-caprolactone (PCL), an FDA approved semi-crystalline aliphatic polymer has been used to encapsulate active drugs. A natural pentacyclic triterpene ursolic acid (UA) was loaded in PCL due to poor aqueous solubility of the free drug.

UA-loaded nanoparticles were prepared through the nanoprecipitation method (Ramanujam et al. 2018).

Nanocapsules

According to a few authors, the use of nano encapsulated compounds is considered a potential approach to enhance the oral absorption and bioavailability of lipophilic compounds (such as nerolidol) in the gastrointestinal tract. The mechanism may be linked with the presence of lactoferrin, a globular protein that acts as a ligand that modifies the structure of nano molecules and crosses the BBB via lactoferrin receptor-mediated transcytosis. In this study, the nanoencapsulation of nerolidol allowed its passage through the BBB, thus facilitating the elimination of *T. evansi* from the CNS. Formulations with trypanocidal action (such as N-NS) have the capacity to cross the BBB and thus eliminate the parasite from the CNS (Baldissera et al. 2017).

Metal Nanoparticles

Another strategy to kill trypanosomes is to target specific protein molecules that are present only in parasites and not in humans. Arginine kinase (AK) which is present in parasites only, is a phosphotransferase enzyme that uses L-arginine and ATP to induce reversible formylation of phosphoarginine. Phosphoarginine acts as a emergency reservoir of ATP and inorganic phosphate. Interaction of gold and silver nanoparticles with recombinant form of arginine kinase obtained from *T. brucei* (TbAK) was investigated. Through spectrofluorimetric study it was found that one binding site of Au and Ag was available for AK with the binding affinities being 43.5 nM and 15.2 nM respectively. To estimate the distance between AuNPs and AgNPs from tryptophan present within TbAK, Fluorescence Resonance Energy Transfer (FRET) was used (Adeyemi and Whiteley 2014). The binding of nanoparticles quenched the fluorescence of tryptophane which was estimated through Försters theory. Försters distance calculated for Ag and Au was 2.18 and 2.35, respectively and according to the literature, if the value lies between 0.5 and 1.5, it indicates high fluorescence transfer taking place.

Emulsions

Sesquiterpenoids are sesquiterpene lactones are obtained from Asteraceae that play a prominent role in human health (Chadwick et al. 2013). Many such compounds like alpha-santonin, arglabin, schkuhrin II, vernolepin, and eucannabinolide have been shown to have an anti-trypanosomal activity. These drugs were loaded into polylactic-acid (PLA) through the emulsion-diffusion method. Even though free compound has greater activity than STL-PAL-NPs but PLA loaded NPs are biodegradable, biocompatible, and have improved pharmacokinetics with the property of prolonged and controlled release (Kimani et al. 2019). The different types of nanoparticles used for treating trypanosomiasis are shown in Table 7.6.

Table 7.6 Types of nanoparticles used against Trypanosoma parasites

Type of nanoparticle	Drug used	Effect
Lipid nanoparticle	4,4-(diazoamino)dibenzamidine (DMZ) + $_{70}DGNP^{+}$	Can enter blood-brain barrier (BBB)
Polymers	Polyethylene glycol + Pentamidine + nanobody	Increase in parasite reduction
	Polysaccharide sodium alginate + Quinapyramine sulfate (QS)	Solubilization and sustained drug release
	Poly-ε-caprolactone + ursolic acid	Controlled release of drug
Nanocapsule	Nerolidol	Ability to cross BBB
Metal nanoparticle	AgNPs	Arginine kinase binds to AgNP and induces formation of phosphoargenine
Emulsions	Sesquiterpenoids + poly-lactic acid	Improved pharmacokinetics

7.5 Toxoplasmosis

Toxoplasmosis is an infectious disease, caused by the obligate intracellular protozoan parasite *Toxoplasma gondii*. Cats are the definitive hosts, with humans, other mammals, and birds being intermediate hosts. Among the three infective stages of *T. gondii*; rapidly dividing tachyzoite, slowly dividing bradyzoite and environmental stage sporozoite; invasive tachyzoite reside in all sort of vertebrate cell type and multiply in the parasitophorous vacuole (Etewa et al. 2018). Treatment targets tachyzoites that disseminate throughout the body. Bradyzoites cannot be affected as they have a low metabolic rate and are protected by the blood-brain barrier (Gaafar et al. 2014).

7.5.1 Current Drugs Available for Treatment

Currently, pyrimethamine is used against *T. gondii* which results in the suppression of bone marrow leading to neutropenia when accompanied by leucovorin supplements. It cannot be used during the first trimester of gestation as folate depletion can have detrimental effects on fetal development. Pyrimethamine in combination with sulfadiazine can cause allergy, kidney stone and hepatic or renal complications. Atovaquone, new dihydrofolate reductase (DHFR) inhibitors such as epiroprim, and antibiotics such as fluoroquinolones cannot be used in pregnant women because of their potentially harmful effects on the embryo (Etewa et al. 2018). Spiramycin has a limited effect due to poor water solubility and dissolution rate (Gaafar et al. 2014). The different drugs currently used for treatment of toxoplasmosis, their mode of action and side effects are tabulated in Table 7.7.

Table 7.7 Current anti-toxoplasmosis drugs, their mode of action and side effects

Drug	Mode of action	Drawback
Pyrimethamine	Inhibits dihydrofolate reductase (DHFR), an enzyme in the redox cycle for the production of tetrahydrofolate required for the synthesis of DNA and proteins (Yaro 2009)	It cannot be used during the first trimester of gestation as folate depletion have detrimental effects on fetal development
Pyrimethamine with sulfadiazine	Inhibition of dihydrofolate synthetase by sulfadiazine and dihydrofolate reductase by pyrimethamine (Yaro 2009)	Allergy, kidney stone, and hepatic and renal complications
Atovaquone (eg. Epiroprim, Fluoroquinolones)	Inhibitor of dihydrofolate reductase (Fry and Pudney 1992)	Developed resistance
Spiramycin	Inhibits protein synthesis by binding to 50S ribosomal subunit (Brisson-Noël et al. 1988)	Poor water solubility and dissolution rate

7.5.2 Use of Nanotechnology in Toxoplasmosis

Polymeric Nanoparticles

Chitosan (CS), a natural polysaccharide, is biocompatible and has two versatile NH_2 groups which make it a capable agent for nanoparticle-mediated drug delivery systems. It possesses the property of biodegradability and is non-toxic. They have mucoadhesive property through which they bind to the mucous membrane and release the drug in a sustained manner (Mohammed et al. 2017). CS has been used against *T. gondii* and has shown mild protection. Administration of AgNPs alone or combined with CS-NPs causes highest IFN-gamma level as a result of enhancement of immunity (Gaafar et al. 2014).

The ability of chitosan to adhere to mucosal surfaces leads to prolongation of its presence at drug adsorption sites and increases drug penetration. Spiramycin loaded chitosan nanoparticles (SLCNs) have been shown to effectively reduced the mortality rate of mice infected with *T. gondii* RH acute and ME49 chronic strain (Gaafar et al. 2014).

Liposomes

Triclosan (TS) and 5-chloro-2-[2,4-dichlorophenoxy] phenol, is reported to reduce the growth of trophozoites in the erythrocytic phase. TS combined with liposomal nanoparticles were tested against *T. gondii* tachyzoites infected mice. TS blocks apicomplexan parasitic replication by binding to Enoyl-Acrl carrier Poritein Reductase (ENR) enzyme and inhibiting its activity (El-Zawawy et al. 2015).

Pyrimethamine (PYR) loaded lipid-core nanocapsules have biological and physical stability and are capable of being administered orally or parenterally. It possessed a high encapsulation efficiency of 92%. Administration of 7.5 mg/kg/day PYR-LNC resulted in increased mice survival rate. It increased bio-distribution of indomethacin

due to enhancement of PYR to the central nervous system, an important site of infection of *T. gondii.*

Metallic Nanoparticles

Given their biocompatibility, gold nanoparticles are of great interest in experimental photothermic therapies which is due to their ability to be conjugated with antibodies and their capacity to strongly absorb light from lasers, allowing them to have utility against infections caused by *T. gondii*. The efficacy of gold nanoparticles conjugated with anti-*T. gondii* antibodies and irradiated with laser light has been shown in a number of studies (Assolini et al. 2017).

7.5.3 Vaccines Developed for Toxoplasmosis

Nanoparticles (NPs) have significant potential as a delivery system. First, the integrity of antigen can be maintained by encapsulating it within nanoparticles or by covalently attaching it to the nanoparticles that protect it from degradation, causes long shelf-life and enhances the possibility of presentation on immune cells (Fang and Zhang 2016). Second, nanoparticle-based antigen systems can deliver antigens to the antigen-presenting cells, such as dendritic cells, by coupling NPs with antibodies that are specific for dendritic cell receptors (Mintern et al. 2013). Third, nanoparticles lower the dose of antigens and adjuvant, thus reducing the risk of toxicity and side effects (Skwarczynski et al. 2010).

Multi-epitope recombinant *T. gondii* vaccine was designed using T and B cell epitopes—SAG1, AMA1, ROP2, and GRA4 proteins have been used based on their binding to major histocompatibility complex. Further, they were encapsulated in poly lactic-co-glycolic acid NPs used as a delivery vehicle. This enhanced the Th1 immune response and significantly reduced the parasite load, prolonging the survival of infected mice (Roozbehani et al. 2018).

Mice were immunized with cocktail DNA vaccines, containing ROM4 which is a serine protease involved in producing high level of humoral and cellular immune response (Han et al. 2017) and GRA14, together with calcium phosphate nanoparticles (CaPNPs), which enhanced the immune response to acute toxoplasmosis (Rahimi et al. 2017).

Adjuvant-free modified dendrimer nanoparticle vaccine simultaneously carries multiple replicon RNAs encoding GRA6, ROP2A, ROP18, SAG1, SAG2A, and AMA1, to protect mice against lethal *T. gondii* challenges (Chahal et al. 2016).

Nanoparticles improve the immunogenicity of nasal vaccines. Porous maltodextrin based with lipid core nanoparticles (DGNP) is used as an antigen delivery system in airway mucosa to form an efficient mucosal vaccine against *T. gondii*. They are formed by enclosing DPPG (1,2-dipalmitoyl-snglycero-3-phosphatidylglycerol) in the core of maltodextrin matrix. The quality of antigen loaded in the nanoparticle is due to ionic interaction and is not affected by DPPG (Dimier-Poisson et al. 2015).

7.6 Amoebiasis

Amoebiasis is caused by *Entamoeba histolytica* which resides it the gastrointestinal tract. It also causes dysentery and liver abscess (Pawar et al. 2016; Zahra'a et al. 2017). Usually, the cyst is transmitted through contaminated food and water. The cyst passes through the stomach and intestine. During this, they lose their cell walls and transform into trophozoites that colonize in the intestinal mucosa. Annually, 40–50 million cases of amoebiasis and up to 100,000 death cases are recorded by WHO worldwide (Zahra'a et al. 2017).

Amoebiasis occurs in two forms: (1) luminal amoebiasis, where no symptoms appear and (2) invasive amoebiasis, where trophozoite invade intestinal mucosa and cause amebic granuloma.

7.6.1 Current Drugs Available for Treatment

A limited number of drugs are found against *Entamoeba histolytica* residing in colonic lumen and tissues leading to invasive amoebiasis. As the drugs are ineffective, combination with other drugs such as paromomycin is recommended. Nitroimidazole derivatives (ornidazole, tinidazole and secnidazole) with a luminal agent (iodoquinol, paromomycin, or diloxanide furoate) could be used to eradicate colonization in case of amoebic colitis (Shirley et al. 2018). A list of current drugs used for treating amoebiasis with their mode of action and side effects are shown in Table 7.8.

7.6.2 Use of Nanotechnology in Amoebiasis

Certain factors responsible for the present drugs not being effective are the solubility of the drug in aqueous and organic media. This is a prime challenge for pharmaceutical companies. Oral administration of such insoluble drugs leads to irregular absorption from the gastrointestinal tract (Pawar et al. 2016).

Nanosuspensions are used to deliver drugs and can be beneficial due to small size and higher stability. Flocculation and Ostwald ripening are also absent in nanoparticles. Atovaquone and Buravaquone are orally administered antibiotics and nanosizing them can increase their bioavailability. The nanosuspension is

Table 7.8 Current antiamoebic drugs, their mode of action and side effects (Swami et al. 1977)

Drug	Mode of action	Drawback
Metronidazole (MTZ)	Inhibits nucleic acid synthesis by disrupting DNA	Not sufficient to eliminate cysts from the intestine
Tinidazole	Generates free nitro radicals	Not advised for asymptomatic amoebiasis Convulsions, diarrhea, hypersensitivity

prepared by the drop-wise addition of the organic phase to the aqueous phase with continuous stirring. In the resultant formulation, the drug release was 95% in 2 h whereas, that of the market product was 85% (Pawar et al. 2016).

Silver nanoparticles, which is one of the most effective metallic particles for treatment has been used against amoebiasis. The mortality rate of parasites for 24 and 48 h almost remained the same, around 50% at concentrations of 50, 75, 100 μg/mL respectively. It was reported that the particle size is inversely proportional to the antiparasitic activity (Zahra'a et al. 2017).

It was reported that Carbon nanotubes (CNTs) and N-doped multi-walled (MW) CNTs were innocuous to trophozoite proliferation and MWCNTs were lethal (Elías et al. 2007).

Bee venom has been used as medicine in various diseases like rheumatic arthritis and cancer but it also showed anti-parasitic activity. Chitosan nanoparticles are nontoxic, crystalline, linear polysaccharide composed of N-acetyl glucosamine and glucosamide. CS NPs possess great binding capacity towards proteins. Further, they are used for the delivery of polypeptides such as snake venoms. To cure amoebiasis, CS NPs were prepared through the ionic gelation method as it is non-toxic and organic solvent-free. The drug release rate was 50% in 5 h and the levels of cytokines IL-6, IL-10 and TNF-alpha were reduced in mice treated with bee venom loaded CS NPs (Saber et al. 2017).

7.7 Cryptosporidiosis

The disease Cryptosporidiosis is transmitted through *Cryptosporidium* oocysts excreted in the feces of infected hosts and contaminated water. There are 14 species of *Cryptosporidium* out of which six are pathogenic to humans and other mammals (Smith and Corcoran 2004). Parasites reside in the intestine, leading to epithelial cell death and malabsorption causing severe diarrhea in acute infection (Gargala 2008). Immunocompromised and HIV-infected people are mainly susceptible to this parasite, making it more evident in developing countries where there is a larger frequency of mal-nourished population (Rossignol 2010). *C. meleagridis, C. felis, C. canis, C. suis, C. hominis* and *C. parvum* species infect humans, from which *C. parvum* and *C. hominis* are predominant.

7.7.1 Current Drugs Available for Treatment

NIH has reported that paromomycin and nitazoxanide have proved to be promising drugs for the treatment of cryptosporidiosis. Apart from that, highly active antiretroviral therapy (HAART) has emerged as a significantly effective therapy for reducing the severity of the disease, particularly in HIV-infected patients. A list of current anti-cryptosporidial drugs with their mode of action and side effects are shown in Table 7.9.

Table 7.9 Current anti-cryptosporidial drugs, their mode of action and side effects

Drug	Mode of action	Drawback
Paromomycin	Targets ribosome and disrupts protein synthesis (Gargala 2008)	Active against *C. parvum* only Infection may relapse (Gargala 2008)
Azithromycin	Interferes with protein synthesis (Gargala 2008)	Does not eradicate the infection completely (Lee et al. 2017)
Nitazoxanide (NTZ)	Inhibition of the pyruvate: ferredoxin/ flavodoxin oxidoreductase (PFOR) cycle	Does not eradicate the infection completely (Lee et al. 2017)

7.7.2 Use of Nanotechnology in Cryptosporidiosis

Silver nanoparticles show toxicity through ion release and generation of oxidative stress. *C. parvum* is sensitive to oxidative stress and is thus destroyed. AgNPs tend to induce oocyst death by disrupting the cell wall. An alternative way to reduce infectivity is the ability of oocysts to undergo excystation i.e. to release sporozoites from host cells. Moreover, the effect of silver ions under these circumstances has also been determined (Cameron et al. 2016).

For long term storage of nanoparticles it is necessary to recover them from suspension into solid state. Spray driying process was used for recovery without altering the dissolution kinetics of active compound. Clofazimine as a potent drug for Cryptosporidiosis, was encapsulated into two biocompatible stabilizers as surface coating, hypromellose acetate succinate (HPMCAS) and lecithin. They were spray-dried through a mini spray drier.

7.8 Conclusion

Nanotherapy has emerged as a boon to combat fatal parasitic infections over the conventional chemotherapies. This therapy involves a range of formulations and has gained popularity due to its precision in administrating medications. Conventional chemotherapies are not target specific, they simply circulate in the body before reaching the affected site. Due to this, a larger amount of drug is required that increases toxicity. Whereas, nanoparticles used directly or encapsulated with drugs have higher specificity, biocompatible, improved bioavailability and require less amount of drug (Mudshinge et al. 2011). Thus, the application of nanotechnology for combating the dreaded parasitic diseases worldwide is absolutely warranted for future therapeutics.

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Concluding Remarks and Future of Nanomedicines

8

Sanjay Singh

Abstract

Nanotechnology and nanoscience have a wide range of applications in drug development, drug delivery, and imaging and treatment of human diseases. Among several strategies, nanoparticle-based drug formulations, nanozymes, nanomaterials-based antimicrobials, scaffolds for tissue growth, and non-invasive cell imaging are some of the recently realized applications nanomedicines. Nanomedicines can be used as personalized medicines and offer reduced toxicity and side effects with concomitant enhanced therapeutic performance. This chapter comprehensively summarizes the recent advances of nanomedicine, associated challenges, and future expectations.

8.1 Nanomedicines

The combination of nanotechnology with biology has led to the novel aspects of approaching better human health and improved biomedical applications (nanomedicine). Recent developments in disease biology linked with genomics and proteomics have offered a significant advantage to nanotechnology to manage various human diseases. To realize the full potential of nanomedicine, research, and developments in several subdomains, such as targeted drug delivery, bio-inspired nanotechnology, cell/tissue imaging, catalytic nanomaterials, and cell/tissue engineering, has been progressed exponentially (Pelaz et al. 2017). Therefore, this book has been designed to comprehensively cover all the frontier areas of nanomedicine research written by global experts.

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Although it has been a few decades since the liposome-based delivery of an anticancer drug (Doxil) and other therapeutic drugs are reported; however, tissue-specific delivery of the intended agent is still infancy. The recent development in the area of molecular and cell biology of the disease has led to identifying specific proteins, nucleotides, and whole cell-based targets of diseased cells/tissues. Similarly, aptamers and siRNA-based approaches for targeting tissues and cell signalling pathways are some of the current and effective strategies that have given desired results when combined with nanotechnology. The route of administration of these nanomedicines in the human body is another point of debate. The oral route of delivery is considered safer than the intravenous; however, the latter is more effective. However, the formation of protein corona (from blood serum proteins) could lead to the alteration in the targeted delivery of drugs. Both of these methods share certain advantages and disadvantages over each other; however, being painless, the oral route of administration is the most preferred method. For chronic therapy and sustained delivery of various anticancer drugs such as Zytiga, Capecitabine, and Topotecan, the oral administration approach has been preferred (Gala et al. 2020). Oral delivery is also preferred for patients because of reduced visits of patients to clinics or venepuncture facilities. The gastrointestinal barrier remains one of the obstacles because the epithelial membrane hinders the permeability and absorption of drugs and the degradation of bioactive components from the formulations. Additionally, in the gastrointestinal tract, the pH variation could be broad from 1–2 (stomach) to 7–8 (colon and rectum). In this context, drugs are encapsulated in the form of nanoparticles, which offer protection of the drugs, leading to an increase in nanocarriers' pharmacokinetics. Some of the best strategies use pH-responsive coating over drugs, nanocarriers of mucoadhesive properties, gastrointestinal enzyme inhibitors, etc. (Zhang and Merlin 2018).

Nanomaterials displaying biological enzyme-like catalytic activities (Nanozymes) are being explored for performing better catalytic activities under the physiological conditions where natural enzymes exhibit compromised activity. Natural enzymes face several limitations: the high cost of synthesis, lower stability, very selective storage condition, poor recycling of catalytic activity, sensitivity to pH and temperature, heavy metals, etc. Based on catalytic activities, nanozymes can be classified into three major types, carbon-based, metal-based, and metal oxide-based nanozymes. These nanozymes are further categorized based on the mimicking activities they display, such as Superoxide dismutase, Catalase, Nuclease, Oxidase, Peroxidase, Phosphotriesterase, and Phosphatase, etc. The antioxidant nanozymes, such as cerium oxide, and platinum nanoparticles, are shown to be used to treat various disorders arising due to the inactivity of the related biological enzymes (Singh 2019).

Similarly, pro-oxidant nanozymes, such as graphene, quantum dots, and iron oxides are shown to produce free radicals, which could be used for several applications such as antimicrobial activity, anticancer effect, and regenerative medicine (Cormode et al. 2018). A combination of multiple materials has led to the development of multifunctional nanozymes equipped with multiple intrinsic properties such as various nanozyme activities associated with magnetism,

luminescence, or near-infrared absorbance. Such multifunctional nanozymes have opened up new industrial opportunities and biomedical applications (Liu et al. 2019). Nanozymes are also shown to be used to develop biosensors to detect biomolecules, disease markers, non-invasive imaging probes, and theranostics. Incorporating nanomaterials in sensors is also expected to create a multifunctional analyzer with enhanced sensitivity and superior performance. Due to these advantages, the incorporation of nanozymes is scheduled to facilitate to develop portable instruments enabling multiplexed analysis of samples even at extremely low concentrations.

Microbial infections with multidrug-resistant species have become a common threat to public health worldwide. Due to antibiotics' excessive use, microbial genome evolves mechanism to survive the antibiotic exposure by developing resistance against it. Due to antibiotics' chemical nature, microbes quickly develop resistance against them; however, exposure of metal-based antibacterial nanomaterials, such as AgNPs, are found to be more effective. Mechanistically, AgNPs bind with the microbial cell wall through noncovalent interaction, and upon entry in the cytoplasm, irreversibly react with "S" and "P" containing biomolecules. Additionally, the biosynthesis of AgNPs leads to the cost-effective and eco-friendly method to synthesize biocompatible AgNPs used for several biomedical applications such as antibacterial, anticancer, antifungal, antiparasitic, antidiabetic and wound healing activities (Wang et al. 2017). Current medical diagnosis requires adequate molecular imaging to facilitate early disease diagnosis, type and stage of the disease, and fundamental information about pathological processes. Non-invasive imaging and diagnosis of the disease is today's need for medical science. This paradigm shift exploits nanomaterial-based imaging probes, which are shown to be better than traditional single molecule-based contrast agents. Quantum dots, fluorophore-doped nanoparticles, radioactively labelled agents, and other nanomaterials have been found to offer excellent results; however, novel nanomaterials as a non-invasive imaging probe for animal/human organ imaging with X-ray based computerized tomography, ultrasound, and magnetic resonance imaging are still being developed (Li et al. 2015). Regenerative medicine and tissue engineering deal with the development of functional human tissues with either model, repairing or replacing the damaged body part to restore, maintain, or improve damaged tissues and organs. Nanomaterials encapsulated in these models and scaffolds have shown excellent outcomes from the in vitro and in vivo experimental studies; however, further thorough research is required for their bench to bedside application. In this context, tissue replacement, such as bone and tooth, and regeneration of damaged tissues/organs with metal or ceramic-based implants and scaffolds are some of the most successful surgical procedures worldwide. Several strategies have been followed to limit the implant failures, such as the type of material, use of composite materials, surface texture, and functional coatings. These strategies are found to have improved the integrity of the implant and normal functioning of the tissues by offering the higher longevity, optimum drug-eluting ability, quicker recovery, limited side effects, etc. The surface coating of implant material with biocompatible functional molecules such as dendrimers has been found to offer minimum implant

failure with improved integrity and tissues' natural functioning. The coating material enhances the implant properties and avoids the identification of implants to the immune system leading to prevent the probability of immune rejection. The globular shape, multivalent nature, presence of a dendrimeric cavity, and the functional versatility of dendrimers are some of the essential factors that allow the formation of a stable soft coating on implants and increase their utility for various biomedical applications (Bai and Liu 2012).

Scaffolds represent a three-dimensional network or structure mimicking the natural extracellular matrix properties and provide structural support to cells/tissues to regenerate. Nanofiber-based scaffolds composed of synthetic polymer biomaterial-based 3D scaffolds have been found to display promising tissue engineering scaffolds that mimic the native extracellular matrix's properties. The encapsulated nanoparticles within the fibers favor cell adhesion, proliferation, and differentiation, which are much needed for the success of tissue engineering applications. Nanofibers have been investigated in multiple tissue engineering applications, including bone, cartilage, ligament, skeletal muscle, skin, and vascular tissue engineering. The scaffolds are also shown to act as carriers for the controlled delivery of drugs, proteins, and genes (Cortez Tornello et al. 2016).

Although there have been several biomedical applications shown by the use of nanomaterials, the associated toxicity must also be unraveled before these nanomedicines are approved for human use. Several research groups have already investigated the toxic potential of various nanomaterials; however, there is no common consensus on synthesis method, capping molecules, test concentration, colloidal stability, aspect ratio, and mono/polydispersity of nanoparticles, etc. Depending on the size of the nanoparticles, they can either be internalized by macrophages (if >100 nm) or taken up into the cell cytoplasm (if <100 nm) by endocytosis and thus associated with higher toxicity risks than the nanomaterials with >100 nm size (Behzadi et al. 2017).

8.2 Associated Challenges and Future Aspects

For delivery applications, nanomedicines are required to be in blood circulation for an extended time so that the effective concentration of the encapsulated compound remains higher. Stealth nanomedicines, coated with biocompatible biomolecules, could offer such advantages without the activation of opsonins. The surface of nanocarriers coated with targeting antibodies or moieties could lead to the selected location delivery of the desired therapeutic molecule. Scaling-up and batch-to-batch reproducibility is another challenge for nanomedicines manufacturing. It is well known that the synthesis of nanomedicines involves multiple steps, such as sonication, emulsification, drying, re-hydration, chemical reaction, and organic solvent evaporation, etc. These processes are easy to control, synthesize, and optimize the formulation at a low volume and small scale level. However, when performed at large scale, slight variations during the manufacturing process may lead to significant changes in the physicochemical characteristics of the nanomedicines with

compromised quality, safety, and therapeutic efficacy. Therefore, a deep understanding of the critical steps of the synthesis and controlled process will be of a great need for the synthesis of nanomedicines at large scale. Similarly, the reproducibility of the physicochemical properties of synthesized nanomedicines is must be ensured by extensive characterization of the product (Soares et al. 2018).

The last decade has witnessed tremendous developments in the translational aspects of several nanomedicines into medical devices and pharmaceuticals. Several nanomedicine discoveries are at the different phases of the clinical trials and are expected to be available for commercial use in a few years. One of the significant limitations in that the discoveries and approvals are country-specific and lack any universally accepted protocol for the characterization, evaluation, and approval for nanomedicines. In this context, the European Medical Agency (EMA) has highlighted the need for suitable recommendations for nanomedicines to facilitate their synthesis, testing, and approval. Further, it is expected that the experts of related fields, including material scientists, biologists, policy and guidelines experts, engineers, managers, and government representatives, must work closely to unravel the real biomedical application potential of nanomedicines.

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