Nanotechnology in the Life Sciences

Ram Prasad *Editor*

Microbial Nanobionics

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Nanotechnology in the Life Sciences

Series Editor

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

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

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Preface

The multidimensional integration between diverse biological kingdoms holds potential toward smart engineering next-generation bionic designs. Bacterial and fungal kingdom exhibits mutual symbiosis that can offer advanced functionalities to bionic architectures. Moreover, functional biogenic nanomaterials can serve as probing agents for accessing new information from microbial organisms due to their dimensional similarities. Microbes are important in human culture, health, and biomedicine in many ways, serving to ferment food, treat sewage, and produce fuel, enzymes, and other bioactive compounds. The use of microbes to synthesize smart biological nanostructures has been of great interest. Microorganisms can change the oxidation state of metals, and these microbial processes have opened up new opportunities for us to explore novel applications, for example, the biosynthesis of metal nanomaterials. In contrast to chemical and physical methods, microbial processes for synthesizing nanomaterials can be achieved in aqueous phase under gentle, nontoxic, cheap, and eco-friendly benign conditions. This approach has become an attractive focus in microbial nanotechnology research toward resource efficiency and sustainable development. This book discusses the synthesis of biogenic nanomaterials by microbes, biomolecules and the mechanisms involved in such biosynthesis, and applications for nanomaterial's of targeted at therapeutics, medicine, agriculture, biomedicine, biofuel and toward new applications that integrate microbes with nanomaterials to produce bio-hybrids and the next generation of bionic architectures.

This book will be immensely useful in the field of bioscience and nanoscience especially to microbiologists, nanotechnologists, researchers, technocrats, and scientists of microbial nanobiotechnology. I am honored that the leading researchers who have extensive, in-depth experience and expertise in microbial system and nanobiotechnology took the time and effort to develop these outstanding chapters. Each chapter is written by internationally recognized researchers/professors, so the reader is given an up-to-date and detailed account of our knowledge of the nanobiotechnology and innumerable applications of microbes.

I wish to thank Eric Stannard, Senior Editor, Springer; Antony Dunlap, Springer Nature, USA; Rahul Sharma, Project Coordinator, Springer Nature; and Kanimozhi Sekar, Project Manager, SPi Global, for their generous assistance, constant support, and patience in initializing the volume. I give special thanks to my exquisite wife, Dr. Avita, for her constant support and motivations in putting everything together. I am also very thankful to Professor Ajit Varma, Amity University, for the constant encouragement. And also, gives special thanks to my esteemed friends & well-wishers and all faculty and colleagues of the School of Environmental Science and Engineering, Sun Yat-Sen University, China, and AIMT, Amity University, India.

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Chapter 1
Introduction to Nanoscience, Nanomaterials, Nanocomposite, Nanopolymer, and Engineering Smart Materials

Yeşeren Saylan, Handan Yavuz, Celal Ülger, Adil Denizli, and Necdet Sağlam

1.1 Introduction: Nanotechnology

Nanotechnology is one of the most important scientific disciplines in which nano-sized (with size of 1 billionth of a meter) materials, tools, and systems are described, produced, and put into use (Bouwmeester et al. 2009). This discipline is not only about miniaturization but also about manipulation of atoms and molecules to control the nanomaterials and/or properties of nanosystems (Serrano et al. 2009). Materials with the size less than 100 nm have some outstanding properties including high surface area, high absorption, dissociation and reactivity, super-paramagnetism, localized surface plasmon resonance, and quantum effects due to their dimensions (Prasad et al. 2016). The development of functional nanomaterials that have many features such as high stability, high performance, and robustness and their use in conventional technologies have led to the emergence of multifunctional nanotechnology-based systems that will accomplish many tasks in one single pass (Saylan et al. 2014).

As shown in Fig. 1.1, there are a number of classes of nanomaterials which are presently used as fluorescent emitters/probes, organic dyes, fluorescent proteins, quantum dots, polymer/dye-based nanoparticles, etc. (Burns et al. 2006).

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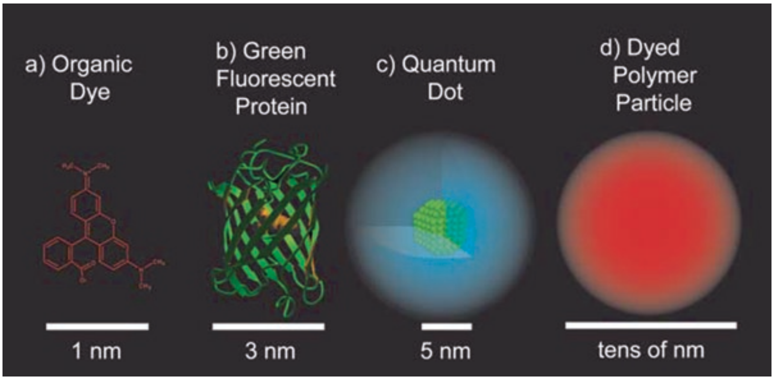

Fig. 1.1 Some examples of nanomaterials: (**a**) Organic dyes, (**b**) green fluorescent protein, (**c**) quantum dots, and (**d**) dyed polymer particles (Burns et al. 2006)

1.1.1 Nanomaterials

Nanotechnology has widely touched the lives of people, which is going to modify most of the aspects and conduct the generation of new products and markets. Nanotechnology is also the multidisciplinary science that makes it difficult to tie down and predict the future effect in any specific area. Research in nanotechnology has increased substantially over the years and all over the world (Bhat 2005).

Although nanotechnology seems to be mostly interested in material science, chemistry, and physics, this discipline has spread to a wide range of life sciences including information science, biotechnology, semiconductors and other technologies. These mutual interactions between science branches are positively affecting many sectors such as space science, textile, ceramics, defense industry, electronics, paper, plastic, renewable and sustainable energy, telecommunications, and automotive (Porter and Youtie 2009).

1.1.2 Microbial Biotechnology

Microorganisms are widely recognized as key markers in the global biogeochemical cycling of nutrients, biomass decomposition, food processing, biofuel production, and waste treatment systems (Suresh et al. 2013). They are also omnipresent in artificial and inartificial ecosystems, and they make up about 30% of earth's total biomass (Ash et al. 2008). They frequently survive as complex areas including multispecies collection dealing an ecological position (Konopka 2009). The effects of nanomaterials on microbial biotechnology have appealed growing research attentions with the prompt development of nanomaterial-microbial interactions due to the impact of the plant and human health (Mohanty et al. 2014; Suman et al. 2010; Singhal et al. 2017).

1.2 Nano-approaches

Nano-based materials have unique physicochemical properties that can be used for many applications in several industries including healthcare, food packaging, water purification, and electronics (Murty et al. 2013; Prasad et al. 2014, 2017a, b, c). According to the latest literature, there are more than a thousand nanomaterial-based products manufactured by at least 500 companies from 20 different countries (Fiorino 2010).

1.2.1 Nanotubes

Nanotubes are one of the most significant nano-based materials with many physical, chemical, and mechanical features. They have several industrial applications such as in medicine, textiles, water treatment, conductor and semiconductor technologies, biotechnology, solar cell, air purification, and mechanical engineering (Hossain et al. 2014).

Giraldo and coworkers showed a single-walled carbon nanotube that transported in passive and irreversibly localized within the lipid envelope of extracted plant chloroplasts (Giraldo et al. 2014). Their single-walled carbon nanotubes encouraged over three times higher photosynthetic activity than controls and improved maximum electron transport rates. They also assembled the single-walled carbon nanotubes-chloroplast enable higher rates of leaf electron transport in vivo through a mechanism consistent with augmented photo-absorption.

Valentini and coworkers created hybrid materials via delivering to unicellular organisms the nutrient to flourish (Valentini et al. 2016). Their system depended on a bionic composite, a combination of carbon nanotubes and *Saccharomyces cerevisiae* yeast extract. They performed tensile analysis on composite films to rationalize a carbon nanotube cell bridging mechanism via the adhesion energy between the matrix and carbon nanotubes. They observed that adding carbon nanotubes also importantly improved the electrical conductivity and photoconductive activity.

Graphene nanoplatelets, carbon nanotubes, and a mixture of these nanomaterials were spread out in silicone rubber with single-celled fungi, carried out by the same research group (Valentini et al. 2017). They performed microorganisms' fermentations while the gelling of the silicone matrix with collapsed cells that deduce as made acceptable with an analytical model and excluded in an abiotic experimental comparing. They also observed an increase in stretchability and damage resistance with respect to the composites made by the abiotic process.

1.2.2 Nanoparticles

Nanoparticles have increasingly been investigated into other matrices. They are intermediate structures between bulk materials and atomic structures. They can be prepared by physical and chemical methods. Nanoparticles are reported to have different associated electronic, magnetic and optical properties and the most reported methods being for metal nanoparticles while other substrates have also been utilized (Weir et al. 2008; Saylan et al. 2017).

Pugazhenthiran et al. isolated *Bacillus* sp., silver resistant, because of the leakage to a silver nitrate solution to the atmosphere (Pugazhenthiran et al. 2009). They first synthesized silver nanoparticles using these airborne bacteria and then confirmed with transmission electron microscopy and energy dispersive X-ray analyses. According to their results, the size of the silver nanoparticles was measured as between 5 and 15 nm, and they were deposited in the periplasmic space of the bacterial cells.

Tilton's research group produced poly-(ethylene oxide) brush-coated nanoparticles with divinylbenzene cross-linked cores and poly-(ethylene oxide) brush shells that have around 30 nm radius and prepared this nanomaterial that biodegraded only suitable coating breakdown mechanism (Kirschling et al. 2011). They supplied poly-(ethylene oxide)-degrading enrichment cultures with either poly-(ethylene oxide) homopolymer or poly-(ethylene oxide) brush nanoparticles as the sole carbon source. They monitored the production of protein and carbon dioxide as quantify of biological conversion.

Dankovich and Gray reported deactivating pathogenic bacteria method via infiltration through a sheet carrying silver nanoparticles (Dankovich and Gray 2011). They characterized the silver nanoparticles and used bacteria inactivation while infiltrating through the silver nanoparticle sheet, instead of bacteria removal from the effluent by filtration. They tested the silver nanoparticles sheet for performance with respect to bacteria inactivation and silver stripping as suspensions of bacteria percolated through the sheet. The silver nanoparticles sheets showed antibacterial properties with ppm levels.

1.2.3 Nanosensors

Nanosensors are compact analytical devices incorporating target molecules as recognition elements nearly related with a transducer to detect an analyte. In general, recognition elements respond to an analyte, and a transducer converts the recognition signal into a measurable response proportional to the analyte concentration (Šefčovičová and Tkac 2015).

A microbial nanosensor for the detection of *Streptomyces*'s viability was reported by Hassan and coworkers (Hassan et al. 2014). They used a carbon paste electrode matrix and studied various nanomaterials' effects on the target organism's viability detection. They optimized a number of factors including the carbon source type, sensing time, scan rate, and cell viability. They observed that the nanosensor was utilized to monitor the growth rate and also the cell viability. They also determined *Streptomyces* response to a beta-lactam which would supply a suitable toxicity determining the system that could be carried out to diagnosis.

Wong and coworkers demonstrated that the spinach plants could be designed to help as pre-concentrators and auto-samplers of analytes in groundwater (Wong et al. 2017). They combined near-infrared fluorescent nanosensors and single-walled carbon nanotubes to detect nitro-aromatics by infrared fluorescent emission.

They accumulated in the mesophyll, resulting in changes in emission intensity. Their nanosensor also allowed to calculate times of residence in the stems, leaves, and roots at 8.3 min and 1.9 min/mm leaf, respectively.

Liu's research group demonstrated an electrochemical nanosensor that depended on gold electrode elaborated by a β-cyclodextrin-incorporated multiwalled carbon nanotube, gold nanoparticles-polyamide amine dendrimer nanocomposites, and chitosan derivative to detect antimicrobial drug chlortetracycline. They first characterized the nanosensor by cyclic voltammetry and amperometry and then carried out the detection experiments in the chlortetracycline concentration range from 9×10^{-8} to 5×10^{-5} mol/L. They also calculated the limit of detection of the nanosensor as 4.95×10^{-8} mol/L (Lian et al. 2012).

Zamaleeva et al. (2011) reported an amperometric whole-cell nanosensor that depended on magnetic retention of living cells functionalized with magnetic nanoparticles. They showed *Chlorella pyrenoidosa* microalgae cells coated with magnetic nanoparticles and kept on the electrode with a permanent magnet for herbicide detection (Fig. 1.2). They observed that magnetic functionalization does not

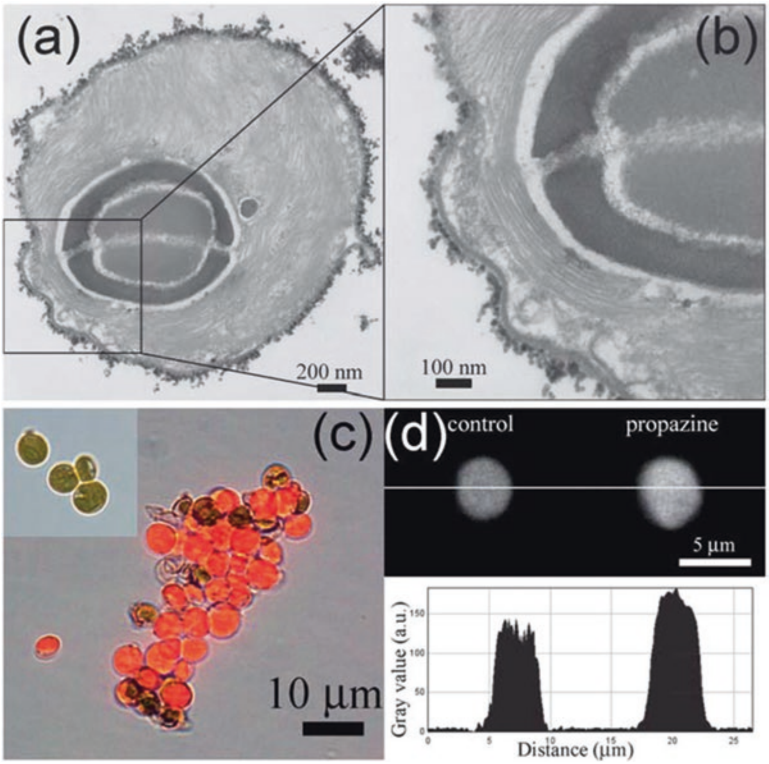

Fig. 1.2 Transmission electron microscopy images of *Chlorella pyrenoidosa* cells coated with magnetic nanoparticles (**a**, **b**), a combined white light and fluorescence microscopy image of magnetically functionalized *C. pyrenoidosa* cells (**c**), a fluorescence microscopy image and a plot got along the line of the control and propazine-treated magnetically functionalized *C. pyrenoidosa* cells (**d**) (Zamaleeva et al. 2011)

influence the viability and photosynthesis activity-mediated herbicide detection in microalgae and also the whole-cell nanosensor detected atrazine and propazine in different concentration ranges and limit of detection values.

1.3 Other Nanomaterials

Parallel with the progress of biotechnology and nanomaterial, a variety of nanointerfaces have been recognized in the biotechnological device design, detection, assays, and other application areas (Wang et al. 2011).

Colpitis and Kiani presented a method for silicon biocompatibility enhancement via production of a three-dimensional nanofibrous layer on the silicon surface, caused by nanosecond laser pulses (Colpitis and Kiani 2016). Their results showed that the laser treatment with a small line spacing and a high overlap number improved the silicon biocompatibility.

Kang and coworkers evaluated the carbon-based nanomaterials' (single-walled and multiwalled carbon nanotubes, colloidal graphites, and aqueous phase C60 nanoparticles) cytotoxicity in Gram-negative and Gram-positive bacteria (Kang et al. 2009). They also evaluated the effect of these nanomaterials on microorganisms in natural and engineered systems. They observed that the single-walled carbon nanotubes inactivated the highest percentage of cells in monocultures and also in the different microbial communities of the river and wastewater.

Zhang et al. employed graphene-based nanomaterial with immobilized bacteria for microbial fuel cells (Zhang et al. 2011). They compared different anode materials by employing cyclic voltammetry. Their results demonstrated higher current responses for graphene-modified stainless steel mesh in comparison with stainless steel mesh and polytetrafluoroethylene-modified stainless steel mesh.

1.4 Application of Nanostructures for Biotechnology and Molecular Genetics

After American biologist James *Watson* and British physicist Francis *Crick* came up with their famous *model* of the *DNA* double helix in the 1950s, many futures of this precious genetic material have been discovered. Nucleic acids (DNA and RNA) are a long molecule that contains genetic code and hold the information of proteins in living organisms and viruses. DNA or deoxyribonucleic acid is the primary molecule for storing biological information. Like DNA, RNA or ribonucleic acid plays an important role in storing information, regulation of gene expression, and biocatalysis. About last 40 years, nucleic acids found a new field of application in material science. They can be designed and manipulated to produce a variety of different nanostructures. Similar to proteins, RNA has a flexible structure and possesses catalytic functions (Guo 2010). Compared with RNA and proteins, DNA molecules are less susceptible to hydrolysis and thus are highly stable. These attributes make DNA a special biopolymer with highly predictable sequence-dependent properties, and these properties have been exploited to construct DNA-based geometric and topologic structures (Lu and Liu 2006). Seeman (2010) pioneered the concept 30 years ago of using DNA as a material for creating nanostructures; this has led to an explosion of knowledge in the now well-established field of DNA nanotechnology. RNA also became attractive after using DNA in order to create nanostructures. The unique properties in terms of free energy, folding, non-canonical base-pairing, base-stacking, and in vivo transcription and processing that distinguish RNA from DNA provide sufficient rationale to regard RNA nanotechnology as its own technological discipline (Guo 2010; Guo et al. 2012). The synthetic nanostructures constructed to date have provided, in addition to affording essential insights into RNA design, important platforms to characterize and validate the structural self-folding and assembly properties of RNA modules or building blocks. Furthermore, RNA nanoparticles have shown great promise for applications in nanomedicine and RNA-based therapeutics (Grabow and Jaeger 2014).

Nanotechnology is having an increasing impact on the healthcare industry, offering unprecedented capability of not only carrying multiple diagnostic/therapeutic payloads in the same “package” but also facilitating the targeted delivery into specific sites and across complex biological barriers (Kang et al. 2005; Farokhzad and Langer 2006; Pardridge 2008; Bonoiu et al. 2009). Gene therapy can be broadly defined as the transfer of defined genetic material to specific target cells of a patient for the ultimate purpose of preventing or altering a particular disease state. Vectors are usually viral, but several non-viral techniques are being used as well. Genes and DNA are now being introduced without the use of vectors, and various techniques are being used to modify the function of genes in vivo without gene transfer (Jain 2008). Nanotechnology-based gene delivery is the division of nanomedicine concerned with the synthesis, characterization, and functionalization of nanomaterials to be used in targeted gene delivery applications. Nanomaterial-based gene delivery systems hold great promise for curing fatal inherited and acquired diseases, including neurological disorders, cancer, cardiovascular diseases, and acquired immunodeficiency syndrome (AIDS). However, their use in clinical applications is still controversial (Keles et al. 2016).

A variety of nanoparticles and other nanostructures can be used for non-viral gene delivery. These include nanoliposomes, gelatin nanoparticles, calcium phosphate nanoparticles, dendrimers, and various composites. Biocompatible, inorganic nanoparticles of carbonate apatite have the unique features essentially required for smart delivery, as well as for the expression of genetic material in mammalian cells. Apatite nanoparticles are promising candidates for non-viral gene delivery and are superior to polymer- or lipid-based systems that are generally non-biodegradable and inefficient (Jain 2008). Artificially synthesized non-bioactive materials are used to deliver nucleic acid materials into cells, which have presented advantages such as low toxicity, low immunoreactions, and the excellent ability to be chemically modified. Yet, these vectors show relatively low transfection efficiency (Sakaguchi et al. 2006; Zhang et al. 2010).

Nanogene vector can combine and concentrate DNA and RNA, effectively introducing the combined or concentrated products into a variety of cells. Through the interaction between phospholipid and glycoprotein with a negative charge on the cations and cell membrane on its surface, nanogene vector enters the cytoplasm, and a positive correlation between the number of cations and the transfer rate of genes can be observed (Jackson et al. 2006). Nanogene vectors, as new drug and gene vectors, present characteristics such as the controlled release, targeting, and the improvement of bioavailability. The satisfying curative effect has been observed from some animal model for tumor. However, most studies are in the stage of in vitro and animal experiments, and in-depth research and human experiments are needed so as to improve the diagnosis rate of tumor at an early stage as well as the curative effect of target treatment (Sun et al. 2014).

Liposomes were the first nanoparticle (NP) platform. Since liposomes were first described in 1965 as a model of cellular membranes, they have moved from a model in biophysical research to one of the first NP to be applied for gene and drug delivery. In one study, NPs were used to selectively silence Cyclin D1, a cell cycle regulatory molecule, in leukocytes in vivo to determine the exact roles of the molecule in gut inflammation (Peer et al. 2008). Another recent report also described the use of lipid NPs for the in vivo delivery of siRNA to silence disease genes in immune cells (Novobrantseva et al. 2012). NPs have also been used as a vehicle to deliver siRNA in plant cells to study cellular pathways at the single-cell level (Silva et al. 2010). Bonoiu et al. (2009) introduced a nanotechnology approach that utilizes gold nanorod-DARPP-32 siRNA complexes (nanoplexes) that target this dopaminergic signaling pathway in the brain. Gene silencing of the nanoplexes in dopaminergic neurons was evidenced by the reduction in the expression of key proteins (DARPP-32, ERK, and PP-1) belonging to this pathway with no observed cytotoxicity. This approach combines the therapeutic potential of gene silencing technology with the imaging and site-specific delivery potential of nanotechnology using gold nanorods.

DNA analysis is widely accepted techniques for personal identification. DNA extraction and isolation from ancient bone samples, degraded body parts, etc. is still quite difficult and challenging task for the forensic scientist. But upcoming technology like nanotechnology will help for analysis of such samples for DNA. In the context of bioseparation and purification, a probable solution is to replace microparticle with nanoparticles as they have a high surface-to-volume ratio. Magnetic nanoparticles have become an increasingly popular technique for the separation of biomolecules, namely proteins, DNA, and RNA. They have been successfully used to extract genomic DNA from body fluids, such as blood, saliva, and semen (Boom et al. 1990). Ansari et al. (2018) reviewed different nanoparticle applications, using magnetic microsphere by Gong and Li (2014) and from cell culture using magnetic nanoparticles by Saiyed and coworkers (Saiyed et al. 2008) and using salicylic acid-coated magnetic nanoparticles by Zhou and coworkers (2013), from soil using silica-magnetic nanoparticles by Sebastianelli and research group (Sebastianelli et al. 2008), from urine using magnetic nanoparticles by Shan et al. (2012) and from saliva using magnetic nanoparticles by Yi et al. (2013). Lodha and coworkers (Lodha et al. 2016) utilized the unique property of copper nanoparticles for DNA isolation from skeletal remains samples.

1.5 Conclusion

Nano-objects from living materials are increasingly being used by nanotechnology. Most of these are produced by microbial cells. Thanks to the advanced and integrated methods like omics and recombinant technologies, production of improved materials with tailored properties for some successful final applications is made possible. The use of microbial cells in factories to supply functional materials for nanotechnology applications is quite expected.

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Chapter 2
Single-Particle Inductively Coupled Plasma Mass Spectrometry for Characterization of Engineered Nanoparticles

Suresh Kaushik, Setyowati Retno Djiwanti, and Efstathia Skotti

2.1 Introduction

With the rapid development of nanotechnology, consumer products containing engineered nanoparticles (ENPs) are commonly available in the market. The most commonly used inorganic nanomaterials in consumer products include metallic nanoparticles such as silver and gold, and metals oxide such as titanium oxide and zinc oxide, as shown in Table 2.1. Nanomaterials with at least one dimension in the nanoscale of 1-100 nm are known as nanoparticles which are released into the environment, and uptake of these nanoparticles is very likely to happen. The concern about the potential risks of ENPs in relation to human health and environment started in 2006 when the World Economic Forum included specific topic "nanoparticle toxicity" in its Global Risk Reports for the first time (World Economic forum, Global Risk 2006). For an extensive and updated list of inorganic nanomaterials, their properties and toxicity, the DaNa (Data and Knowledge on Nanomaterials) site is recommended (Tuoriniemi et al. 2012).

The release of free NPs from the embedded NPs in a solid matrix or ionic components from the dissolved NPs must also be considered because all these physical and chemical properties are closely responsible for the occurrence of fate and toxicity of these NPs (Donovan et al. 2018). The current challenge for the analytical scientist is to develop innovative approaches to detect, characterize and quantify

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Table 2.1 Some examples of consumer products, environmental and tissue samples containing ENPs

Name of products or samples	Nanoparticles
Moisturizing cream	TiO_2
Hand sanitizer gel fabric	Ag
Sunscreens	TiO_2, ZnO
Disinfectants	Ag, ZnO
Antibacterial products	Ag
Food personal products	Au, Ag
Dietary supplements	Ag, Au, Zn, Si, Cu
Coffee creamer	SiO_2
Washing water	Ag
River water	Ag
Tap and seawater	Ag

ENPs in complex samples at realistic concentrations and in presence of natural particles of similar or different nature. The reason for the lack of analytical methods is related to the unique physical and chemical nature of NPs as solid analytes.

Complete characterization of nanomaterials is important for interpreting the results of toxicological and human health studies (Witzles et al. 2018). Metal-containing nanoparticles are particularly significant class because their use in consumer and industrial applications makes them the fastest growing category of NPs. Hence, innovative analytical approaches are essential for monitoring the presence of nanomaterials in environmental and biological media, assessing their potential impact and supporting regulations.

Many analytical techniques are available for nanometrology, only some of which can be successfully applied to environmental health studies. Methods for detecting, quantifying and characterizing these materials in complex matrices are critical for the eventual understanding of their implications to environmental quality and human health. Methods for assessing particle size distribution include electron microscopy, chromatography, laser-light scattering, ultrafiltration, and field-flow fractionation. Common approaches used to characterize nanomaterials include optical properties methods, e.g., dynamic light scattering (DLS), and microscopy-based methods, e.g., transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM).

A well-established technique of inductively coupled plasma mass spectrometry (ICP-MS) has been around for several decades for elemental analysis, but it is not capable of providing any information about the physicochemical form of the element (e.g., present as dissolved species or as particulate) or any other information pertaining to the ENPs (e.g., shape, size, aggregation, etc.). Recently, single-particle ICP-MS has emerged (spICP-MS) as a useful tool for characterization of metal-containing nanoparticles. This technique is reliable and fast for counting and sizing particles at lowest concentrations while simultaneously distinguishing between dissolved and particulate analytes. Additional information about the soluble species or the nanoparticles can be obtained, if ICP-MS is coupled with a previous separation step. Hence, information about size and quantification of nanoparticles can be obtained if ICP-MS (element-specific detector) is coupled to continuous separation techniques such as field-flow fractionation (FFF) or hydrodynamic chromatography (HDC). In this chapter, development of single-particle ICP-MS technique hyphenated with nanoparticles size separation methods from niche techniques to application for routine analysis will be reviewed.

2.2 ENPs and Their Characteristics

Research on metallic ENPs having at least one dimension in the range of 1–100 nm has gathered momentum because of their potential for a diverse array of applications in science, technology, and medicine (Webster 2006). Some examples include titanium dioxide NPs, gold NPs, platinum NPs, silver NPs, and quantum dots (Duncan and Gaspar 2011). According to the Nanotechnology Characterization Laboratory (NCL) at the National Cancer Institute, National Institutes of Health (NIH), NPs have a size range between 1 and 100 nm (McNeil 2005). ENPs with dimensions in the nanometer scale are typically intermediate between isolated small molecules and bulk materials. Compared to their bulk material counterparts, the distinct physiological properties of the NMs such as size, shape, surface properties, composition, molecular weight, identity, purity, stability and solubility are critically relevant to particular physiological interaction (Patri et al. 2006).

Size defined as an equidistant diameter of a spherical particle is a crucial factor in ENMs whose physical properties are equivalent to those of the NMs in the same environment (Powers et al. 2006; Shekunov et al. 2007). The shape of ENPs can play an important role in drug delivery, degradation, transport, targeting and internalization (Champion et al. 2007; Decuzzi et al. 2009).The shape of NMs affects uptake, biocompatibility and retention in tissue and organs (George et al. 2012). NMs are characterized by a relatively large surface area per unit mass. As the surface area of a solid depends on its shape and size, the change in size or shape of NMs can affect their physicochemical and physiological properties. In addition to size and shape, chemical composition is another important factor in determining toxicity of NPs (Buzea et al. 2007). Other properties such as surface energy, wettability, surface charge, and species absorbance or adhesion are commonly considered important parameters (Brodbeck et al. 2001; Patri et al. 2006). While surface composition is relevant to the superficial layers, surface energy involves the aggregation, dissolution and accumulation of NM. Surface charge governs the aggregation and dispersion stability of NMs. Species absorbance or adhesion potentially alters the surface of NM as well as the conformation and the activity of the attached species (Ratner et al. 2004). As the composition of NMs affects delivery, transport, and biodistribution, a variety of NMs are utilized in the production of potential nanomaterials and nanomedicines which can be categorized by their structural types such as nanoparticles and its derivatives, quantum dots, emulsion, micelle, liposomes, dendrimers, fullers, carbon nanotubes, and hydrogels. Sometimes, there may be a need to combine two or more types of NMs to form a complex such as conjugate, a chelate. In such cases, chemical analysis of the NM complex is more complicated

Table 2.2 Some analytical techniques for physicochemical characterization of ENPs

Nanomaterial characters	Technique used
Size/size distribution	Dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), FFF (field-flow fractionation)
Shape	SEM, TEM, attenuated total reflection Fourier transform infrared (ATR-FTIR), electrophoretic light scattering (ELS) for zeta potential
Structure	Circular dichroism (CD), mass spectrometry (MS), infrared (IR), scanning tunneling microscopy (STM), XRD
Surface charge	ATR-FTIR, ELS, capillary electrophoresis (CE)
Composition	MS, nuclear magnetic resonance (NMR)
Purity	HDC, high-performance liquid chromatography (HPLC), NMR
Stability	Thermal gravimetric analysis (TGA), differential scanning calorimetry (DSC), HDC, HPLC
Surface properties Dispersion	ATR-FTIR, CD, X-ray photoelectron spectroscopy TEM, STM, atomic force microscopy (AFM), environmental SEM (ESEM)

Source: Lin et al. (2014)

than that of a single entity (Patri et al. 2006). There are several studies addressing toxicological concern about NPs of different composition (Hardman 2006).

Nanomaterial stability refers to retaining the same properties for a period of time after the NM is manufactured. The stability may be affected by one or more factors such as moisture, temperature, pH, particles size, enzymatic degradation, and solvents (Briscoe and Hage 2009). This property may also impact its corresponding toxicity (Hardman 2006).

The surface properties of NMs in a given medium are characterized by their physicochemical properties such as shape, surface geometry, chemical composition, heterogeneity, porosity and hydrolytic stability. Ionic strength, temperature, pH and presence of organic macromolecules affect other properties such as dissolution, surface charge, size distribution, hydration, dispersion stability, agglomeration and aggregation of NMs (French et al. 2009).

Thus, physicochemical characterization of NMs should be profiled based on different physical state of the ENPs (suspension, solution, or dry powder), and therefore, techniques for determining the shelf-life of ENM formulations are essential. Various techniques are available (Table 2.2) to detect, characterize, and quantify engineered nanomaterials (Lin et al. 2014; Laborda et al. 2014, 2016).

2.3 Analytical Techniques for Characterization of ENPs

The information demanded for commercial analytes is qualitative (identification of chemical species) and quantitative, but when the analyte is an ENP (a solid-phase species), the quantitative information such as mass or molar concentration and number concentration as well as qualitative information such as detection of the ENP is demanded. Moreover, chemical (core and coating composition) and physical (size, shape, aggregation/agglomeration) characterization can be demanded for an ENP (Fig. 2.1).

Methods and techniques to characterize ENPs in simple matrices were already available, but methods to qualitatively trace ENPs released into environmental system were rarely available (Howard 2009). The reason for this lack of analytical methods is related to the unique physical and chemical nature of NPs as solid analytes. The release of free NPs from the embedded NPs in a solid matrix or ionic component from the dissolved NPs must also be considered. All these physical and chemical properties are closely responsible for the fate and toxicity of NPs. The current challenge for the analytical scientists is to develop innovative approaches to detect, characterize, and quantify ENPs in complex samples, at realistic concentration and in the presence of natural particles, of similar or different nature. Electron microscopy, light scattering techniques, atomic spectrometry, chromatography and non-chromatography continuous separation techniques, electroanalytical techniques, X-ray scattering, centrifugal particle sedimentation, and analytical ultracentrifugation are numerous analytical techniques and methods for detection, characterization, and quantification of ENMs. There are various types of light scattering techniques such as dynamic light scattering (DLS), static light scattering (multiangle light scattering), and nanoparticle tracking analysis (NTA). DLS, also

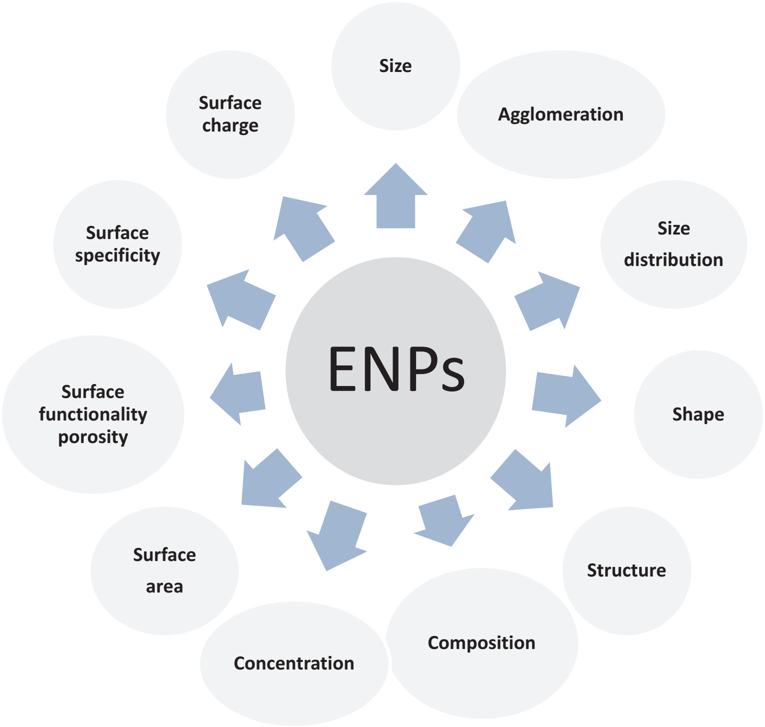


Fig. 2.1 Key parameters to characterize ENPs

known as photon correlation spectroscopy, measures the Brownian motion of NPs, through the time-dependent fluctuations in scattering intensity caused by constructive and destructive interference. This movement is related to an equivalent hydrodynamic diameter (Brar and Verma 2011). The static light scattering provides measurement of physical properties which are derived from the angular dependency of the light scattered by NP. Similarly, NTA is the technique in which the movement of particle under Brownian motion is measured by using video microscopy, and their hydrodynamic diameters are calculated using a modified Stokes-Einstein equation (Gallego-Urre et al. 2011).

Electron microscopy is the most used and powerful technique for measuring the size and all information about the shape and aggregation state of NPs (Hendrickson et al. 2011; Sadik et al. 2014). In EM, sample preparation and high-vacuum conditions are key features to achieve a good characterization of ENPs. Conventional TEM is used for characterization of ENMs in complex matrices and can provide precise particle size information at nanoscale. The introduction of field-emission electron gun in scanning EM has improved the better spatial resolution (<1 nm), enabling this type of instrument for the successful use of scanning instrument for ENPs characterization.

X-ray absorption spectroscopy (XAS) is an element-specific technique providing specific quantitative information about metal/metalloid species. This technique provides the information about their quantitative distribution but not about their particulate nature in complex liquid and solid samples such as tissues, soil, and sediments. In this technique, there is no need of sample preparation, and moreover, the physical and chemical original states are preserved. There are two types of XAS, namely XANES (X-ray absorption near edge structure), which provides information about the geometry and oxidation states, and EXAFS (extended X-ray absorption fine structure), which provides information about the element coordination. The main demerits are sensitivity of the technique, which is in the range of mg/kg, and difficulty to interpret the bulk data when sample consists of a complex of species.

There are several element-specific techniques such as electrothermal atomic absorption (ET-AAS), ICP-OES, and ICP-MS used to achieve this objective. The first step in the analysis of a complex sample involves the detection of an inorganic ENM. ET-AAS allows the introduction of samples both in solid and liquid phases (solution and suspension). ICP-MS is one of the most common techniques for the identification of inorganic ENMs due to the low detection limits (ng/L). ICP-based techniques involve the use of liquid phases. Hence, solid samples must undergo some sample treatment for the digestion of matrix. Suspension could be directly analyzed. The feasibility of direct analysis of suspension by ICP-MS depends on the size and composition of the NPs (Lee and Chan 2015). These techniques can be used for sensitive detection and quantification of the elements present in the ENM and the samples (Krystek et al. 2011). A number of research articles have been published describing the state of the art of analytical methods for the detection, characterization, and quantification of ENPs (Hasselov et al. 2008; Tiede et al. 2008; Marquis et al. 2009; Farre et al. 2009; Howard 2009; Yu and Andriola 2010; Fedorov et al. 2011; Calzolai et al. 2012). Although a number of techniques for characterization of ENPs are available, their application can be unfeasible in most cases when moving to complex situations and working at trace levels. Some emergent and promising techniques (Lin et al. 2014) which are commonly used are listed in Table 2.2. The technique that is proving invaluable for detecting and sizing metallic nanoparticles is single-particle inductively coupled mass spectrometry.

2.4 ICP-MS and spICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) is a powerful analytical tool (Hu et al. 2018; Bustos and Winchester 2016) that allows for accurate determination of sample metal content at concentration in the low microgram per liter or even nanogram per liter range. An ICP-MS combines a high-temperature ICP (Inductively Coupled Plasma) source with a mass spectrometer. The ICP source converts the atoms of the elements in the sample to ions. These ions are then separated and detected by the mass spectrometer. ICP-MS is undoubtedly the fastest growing trace element technique available today. The high sensitivity of the instrument allows for detection at the parts-per-trillion (ppt) level. The ability to carry out rapid multielement determination at the ultra-trace level has made it very popular in diverse range of application areas including environment, geochemical, semiconductor, metallurgical, nuclear, chemical, climatic, and biotechnology. In recent years, industrial and biological monitoring has presented major need for metal analysis by ICP-MS (Fig. 2.2). Other applications of this technique are in the medical and forensic field, specifically, toxicology and heavy metal poisoning study. Hence, spICP-MS is an emergent ICP-MS method for detecting, characterizing and quantifying nanomaterials as shown in Fig. 2.3. This can be considered one of the innovative and emerging analytical approaches to provide information about the elemental chemical composition of nanomaterials as well as their number concentration, size

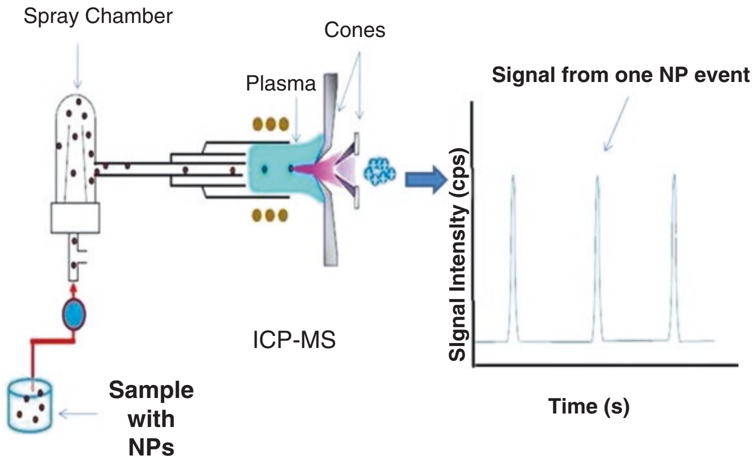


Fig. 2.2 An outline of single-particle analysis by ICP-MS

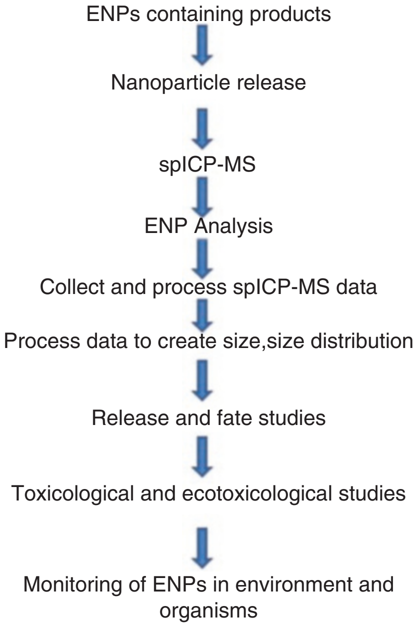
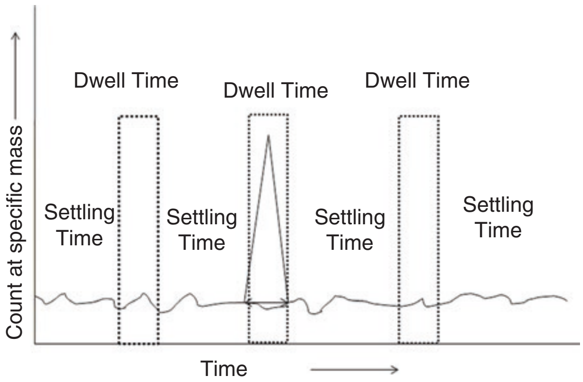


Fig. 2.3 Flow diagram of the process to characterize ENPs using spICP-MS for monitoring their fate in environment and ecotoxicological studies

and number size distribution. This is based on the measurement of the signal intensity produced by a single particle. Nanoparticle suspensions are sufficiently diluted to minimize the chances that more than one particle reaches the plasma at a time. The particle is atomized and ionized, producing a signal of relatively high intensity which is measured as a pulse, whereas the dissolved analytes contribute to continuous signal (Fig. 2.4). The frequency of pulses (events) provides the particle number concentration, whereas the intensity of each pulse is proportional to the mass of the particle. Because of the short transient nature of the pulse, very short integration times are necessary to maximize the detection of individual particles as pulse of ions after they are ionized by the plasma.

Its combination of elemental specificity, sizing resolution, and unmatched sensitivity makes it extremely applicable for the characterization of nanoparticles containing elements such as titanium, gold, silver, and silicon, which have been integrated into larger products such as foods, consumer goods, personal care products, and pharmaceuticals. The technique was initially adopted for analysis of aerosol and airborne particles. Subsequently, this methodology was implemented for study of colloidal and microparticle suspensions. More recently, the interest on spICP-MS as an alternative to other available techniques for detection, determination, and characterization of nanomaterials has been renewed and increased for the characterization of ENPs to monitor the fate of nanoparticles in the environmental and health issues as depicted in Fig. 2.3.

Fig. 2.4 A continuous signal for a metallic element, showing the dwell and settling times, but data are collected only during the dwell time window (Source: Stephen and Thomas, 2017)



2.5 Basic Principles for Characterization of ENPs by spICP-MS

The basic assumption behind spICP-MS is that each recorded pulse represents a single NP. A very dilute suspension is introduced into the ICP-MS instrument, such that statistically only one nanoparticle at a time enters the plasma. For effective detection and measurement of individual and single nanoparticle, ICP-MS instrument is operated in different manner as compared to analyzing the dissolved samples. spICP-MS involves introducing nanoparticle-containing samples of environmentally significant concentrations into the ICP-MS system and collecting time-resolved data. Due to the very low elemental concentrations and the transient nature of ionized nanoparticle, very short measurement times and high sensitivity are essential to ensure the detection of individual particles as ion pulses. The number of observed pulses at the detector is related to the nanoparticle concentration by the nebulization efficiency and the total number of nanoparticles in the sample, while the size of the nanoparticle is related to the pulse intensity.

Figure 2.4 shows traces from both single NP and dissolved analyses. A steady-state signal represents the dissolved elements, and each spike signal represents a particle. The difference in a way these data are produced plays a key role in understanding single-particle analysis (Stephen and Thomas 2017). When dissolved elements are measured, aerosol enters the plasma and the droplets are desolvated and ionized. The resulting ions enter the quadrupole to be sorted by their mass-to-charge ratio (m/z). The quadrupole spends a certain amount of time at each mass before moving to the next mass; the time spent analyzing each mass is known as "dwell time." After each dwell time measurement, a certain amount of time is spent for the electronics to stabilize before the next measurement is carried out. This stabilization is known as "settling time." This is also called overhead and processing time. When analyzing dissolved elements, the resulting signal is essentially a steady-state signal. But when particles present in an aqueous solution are introduced to the plasma, a burst of ions (one ion cloud per particle) after the droplets' desolvation and particle ionization processes in the plasma. The ions then pass into the quadrupole. Ion clouds are not always detected during alternating between dwell time and settling time. If the ion cloud happens to fall within the dwell time window, it will be detected; otherwise, it will not be detected as shown in Fig. 2.4.

In spICP-MS, transient data acquisition speed consists of two parameters: dwell time (reading time) and settling time (overhead and processing time). It is very important that ICP-MS should be able to acquire signals at a dwell time which is shorter than the particle transient time. The shorter the settling time, the less chance there is of missing a particle. Dwell time of 50–100 microseconds is generally considered, but recently, Stephen and Thomas (2017) reported that analysis of samples using spICP-MS can be performed using ICP-MS instrument capable of collecting data at or below 10 microseconds.

As ICP-MS is basically a mass-based technique, in spICP-MS, particle size is determined by relating the pulse intensity to an elemental mass. In ICP-MS analysis, a calibration curve using dissolved standards is created which connects the signal intensity from the instrument to the concentration of the analyte entering the plasma. Subsequently, the concentration of the dissolved analyte to a total analyte mass that enters the plasma during each reading is related. This relationship between analyte concentration and the mass observed per event is known as the "mass flux." This mass flux is highly dependent on the transport efficiency of the sampling process, which must be calculated for each instrument and under the run conditions for the mass flux to be accurate. Hence, the calibration curve relates signal intensity (counts/event) to a total mass transported into the plasma per event. The intensity of each individual pulse (counts/event) can then be transformed using the mass flux calibration curve to determine the particle mass, which can then easily be converted to particle diameter by knowing the density and shape (assuming spherical) of the particle.

The parameters of the nanoparticle population then can be measured including the mean size, size distribution, NP number concentration, and NP mass concentration. As the frequency of the pulses is directly related to the number concentration of NPs, the intensity of each pulse is proportional to the mass of elements, in fact, to the number of atoms, in each detected NP.

2.5.1 Size of ENPs

In single-particle analysis, the detection of NP size depends on two things: their number concentrations and the size or the element mass per NP. The number concentration should be high enough to allow counting a minimum number of events. It means that their detection is associated to the capability of identifying the pulses from the NPs over the baseline produced by the continuous background in a time scan. The smallest pulse height which is distinct from the background determines the smallest detectable NP mass, i.e., the smallest detectable NP size. These NP pulses can be identified in a frequency histogram. The minimum particle that can be determined by this method is strictly linked by signal to background. Background is composed of instrumental noise, spectral interferences and dissolved ions concentration.

Pace et al. (2012) described a method of sizing analysis using analysis of a NP standard to produce a mass flux curve from a dissolved standards concentration. The mass of the analyte in the NP can be determined by relating the measured intensity to the mass delivered by the dissolved standards. Concentration, sample flow rate, and dwell time are all user-defined, but the transport or nebulization efficiency must be determined experimentally. Pace et al. (2012) described methods for determining this value. If the nanoparticle analyzed consists of more than one element, the mass fraction is used to convert the signal from the singular element to an overall mass of particles.

2.5.2 Number Concentration of ENPs

The frequency of NP events (N) counted is directly related to the NP number concentration, and number of events (N) for a fixed nanoparticle number concentration (N_{NP}) can be increased proportionally by increasing the total acquisition time (t). By improving the nebulization efficiency, increasing the sample flow rate and/or using longer acquisition times, number concentration detection limits can be enhanced. Because number concentrations are not dependent on the NP nature, number concentration calibrations are performed by using spherical, solid, and pure NP suspensions of known average diameter and mass concentrations (e.g., NIST RM 8103) for the quantification of any type of suspension.

2.5.3 Dissolved and Nanoparticle Forms of ENPs

Specific information about the occurrence of dissolved and nanoparticles forms of the element can be achieved, if the time scan is processed as a frequency distribution histogram as shown in Fig. 2.4. If both distributions are fully resolved, the first one displays a Poisson profile and other one is similar to size distribution of NPs. The element mass concentration in a NP suspension can be determined by working in standard mode, but mass concentration can be determined in single-particle mode by summing up the pulse integrity of the events recorded or by integrating the corresponding histogram, i.e., sum of the pulse intensities multiplied by the number of pulses. The content of the element as dissolved or NPs forms can be achieved in the forms of mass concentration on integration for different distribution obtained in histogram, if both distributions are fully resolved.

2.6 Sample Preparation of ENMs

Most samples before analysis require some kind of preparation by different analytical techniques and methods. The same case is with ENPs' sample preparation before characterization by analytical techniques and methods involving removing the matrix or separating the ENMs from matrix. spICP-MS technique requires aqueous solution for particle size analysis; thus, solid media require extraction prior to nanoparticle determination. For total metal analysis, acid extraction techniques are generally sufficient, yet the aggressive reagents used destroy the nanoparticles, producing dissolved ionic species. ENMs can be extracted from solid and liquid samples by using water and organic solvents preserving some of the properties of the ENMs.

As spICP-MS is based on introducing individual ENPs into the plasma, this technique requires dilute solution so as to avoid particle coincidence. Hence, it is most applicable in the low to mid ng/L range and is thus ideal for analyzing ENPs' samples (Pace et al. 2011; von der Kammer et al. 2012). Nanoparticles in solid media (tissue, sedimentation, and soil) require extraction of the solid media to an aqueous phase prior to analysis with subsequent dilution to analyte concentration. When nanoparticles in solutions are characterized, nanoparticle concentration is primary limiting factor. Tetramethylammonium hydroxide (TMAH), a strong base, has been used on tissue samples containing multiple ENPs sizes as well as samples containing both dissolved and ENPs (Gray et al. 2013). This reagent TMAH does not impact the primary particle size of ENPs as compared to the particle analyzed in water.

Recently, cloud point extraction (CPE) has been proposed for the separation of NPs, preserving the size and morphology of the NPs in sample. This provides selective separation of the NPs from dissolved species (Liu et al. 2009a). This extraction technique involves the addition of a non-ionic surfactant (Triton X114) at concentration over the critical micellar concentration. CPE has been combined with ET-AAS (Hartmann and Schuster 2013; Hartmann et al. 2013; Lopez-Garcia et al. 2014) and ICP-MS (Liu et al. 2009b; Majedi et al. 2012, 2014) for quantification of NPs. This strategy can be combined with total element determination to obtain information about the dissolved and nanoparticulate element. Anionic exchange resins have been proposed for the selective extraction of noble metal NPs (Li et al. 2012).

The concentration of NPs in environmental and biological samples may be expected to be lower than those that can be directly measured. So, it is necessary to remove matrix components. Separation in combination with pre-concentration can be useful to improve the detection capability of the measurement techniques. Centrifugation can be considered the simplest approach to isolate particulates from an aqueous suspension as well as to separate NPs from dissolved species (Unrine et al. 2012). Serial filtration in combination with ultrafiltration has been used for sized fractionation, followed by characterization and quantification of each fraction by a number of techniques and methods (Mitrano et al. 2014).

2.7 Hyphenation of spICP-MS for Size Separation

spICP-MS is just able to measure the elements present in a particle. This means that two different NPs with the same element content behave in the same way in spICP-MS. Similarly, NPs of different shapes or NPs with the same-shape core diameter but different coating concentration may also behave in the same way. ICP-MS in single-particle mode has potential to increase the available information from a single analysis by combining initial separation techniques flow-field flow (FFF) or capillary electrophoresis (CE). Field-flow fractionation is used as the separation step because it is well suited to the mass and size range of NPs.

The principle of FFF was developed by Calvin Giddings in 1966 as shown in Fig. 2.5 (http://www.postnova.com/general-theory.html). In this technique, separation is carried out in an empty channel by applying an external separation field perpendicular to the sample flow. Different sized particles diffuse back against this field at different rates leading to separation of the particles in the different flow layers. The different layers travel at different velocities, resulting in the sequential emergence of analyte molecular or particles. The smallest particles emerge first and the fastest as shown in Fig. 2.5. The ICP-MS can be used as an elemental detector for separation techniques such as field-flow fractionation.

Field-flow fractionation consists of suite of high-resolution sizing techniques, which allow separation and sizing of macromolecules, submicron colloids and nanoparticle of 1–100 nm, depending on the type of field applied and mode of operation (Ranville et al. 1999). The FFF technique depending on the type of analysis to be performed is chosen to achieve optimal separation results. FFF is a developing online fractionation technique that enables the separation of macromolecules, colloids, nano- and microparticles according to size, chemical composition, or density with excellent resolution over a size range from a few nanometers up

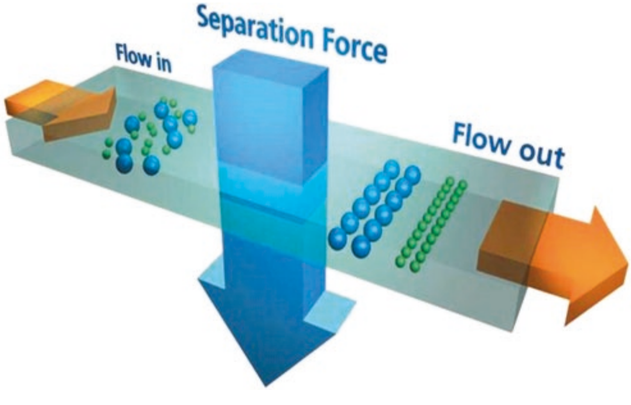


Fig. 2.5 Basic principle of field-flow fractionation. (Source: Stephen and Thomas 2017)

to several microns. There are many demerits of other separation methods such as degradation, filtration and unwanted adsorption which can be overcome by field-flow fractionation.

Among hyphenated techniques with ICP-MS as the detector, field-flow fractionation served as completion choice to characterize NPs due to the mild separation, high selectivity, and broad application in continuous size separation range. Among the FFF techniques, flow FFF is considered as the most universal due to its simplicity in instrumentation and operation in various analyses. Hence, flow FFF coupled with ICP-MS is regarded as one of the most promising tools to characterize NPs, and Lee et al. (2016) described the potential applicability of flow FFF offline hyphenated with spICP-MS in characterizing NPs in real aqueous samples.

The hyphenation of asymmetric flow-field flow fractionation (AF4, a type of flow FFF) and ICP-MS has proved to be a suitable technique for NPs' separation and characterization in various matrices. An emerging variant of flow FFF, hollow fiber flow field-flow fractionation (HF5), has simple assembly with low cost in disposable channels, resulting in the reduced run-to-run sample carryover problem from the sample matrix. In HF5, separation is conducted in a cylindrical hollow fiber membrane, and the flow field is applied perpendicular to the hollow fiber axial direction, while in conventional flow FFF, thin ribbon-like open channel is used. As compared with the AF4 channel, less sample dilution is needed in the low channel volume of HF5 channel. HF5 is more compatible with ICP-MS due to the reduced radical flow rate.

Capillary electrophoresis (CE) and hydrodynamic chromatography (HDC) are two separation techniques which separate particles based on their diameter. When coupled to spICP-MS, these techniques can provide an additional selectivity. HDC has been coupled online to spICP-MS till now (Pergantis et al. 2012). Loeschner et al. (2013) have measured fractions from enzymatically digested meat samples spiked with AgNPs and collected from separation performed by asymmetrical flow field-flow fractionation (AF4)-spICP-MS to obtain the corresponding number size distributions and to detect the occurrence of dissolved Ag. The CE coupled to ICP-MS has been used to separate a range of very small metallic NPs in dietary supplements. CE-ICP-MS has the potential benefits of much shorter run times and smaller sample size requirements.

Ion-mobility spectrometry (IMS) technique based on a combination of mass, charge, size, and shape of ionize species has been used to analyze macromolecules, viruses, and NPs by means of condensation courting detectors. Recently, nanoelectrospray has been offline coupled to a spICP-MS to demonstrate the feasibility of the hyphenated technique for element-specific size determination of AgNPs (Kapellios and Pergantis 2012).

The early work has focused on the use of ICP-MS with particle separation techniques (chromatography and field-flow fractionation). These hyphenated separation techniques can be further improved and developed. However, more recently, this spICP-MS technique is applied to differentiate between the analyte in solution and exiting as a NP without any prior separation techniques, thus simplifying NP analysis and eliminating complex sample preparation steps. Hence, spICP-MS can provide information on the size and size distribution of NPs as well as the concentration of the analyte.

2.8 Strength and Limitations of spICP-MS

The strength of this technique is that it can be performed using off-the-shelf ICP-MS instrumentation. There is no need of instrumentation modification. Some additional instrument capabilities such as very short detector dwell time on the order of microseconds, automated data reduction software, and hyphenation with a frontend separation technique such as field-flow fractionation may be further developed. Another important strength of spICP-MS is its superb detection capability in terms of NP number or mass concentration. The technique can be readily used to detect and characterize NPs at the extremely low number concentration (in the range of 10^3 cm^{-3} to 10^5 cm^{-3}) which are found in real-world environmental samples.

There are shortcomings of this technique as well. The technique can measure only one or at most two isotopes in a single analysis using quadrupole instrument, the most common type of ICP-MS instrument. The detection power in terms of NP size is still lacking. The typical limit of detection (LOD) is expressed as equivalent spherical diameter ranging from 10 nm to 20 nm for monoisotopic NPs.

Hence, this technique is suitable for NPs consisting of one element only and size higher than 20 nm. So, improved spICP-MS instruments are needed with enhanced size detective limits and multielemental capability for heterogeneous NPs. The multielement detection of transient signal generated by individual NPs involves the use of simultaneous or fast scanning instruments which are able to record full spectra of a mass range of interest at frequency around 10^5 Hz (Engelhard 2011). The currently available instruments do not permit the continuous monitoring at acquisition time below several terms of milliseconds (Borovinskaya et al. 2013), which is not useful for multielement spICP-MS. Recently, Borovinskaya et al. (2013) have developed prototype TOF-ICP-MS which is capable of quasi-simultaneous multielement detection at acquisition times of 33 microseconds and with detection efficiency similar to those of quadrupole-based instrument.

Single collector sector field and quadrupole instruments working in single-particle mode are used to monitor just one isotope because of the sequential scanning nature of these mass analyzers. Recently, fast scanning modes have been proposed for both sector field and quadrupole instruments (Badiei and Kajen 2013). An acquisition time of 100 microseconds has been attained with a commercial sector field ICP-MS instrument, whereas acquisition time quadrupole down to one microsecond using quadrupole ICP-MS has been reported. But, attainable settling times between isotopes can be limitations for multielement detection in a single nanoparticle.

Pneumatic nebulization systems are used for liquid samples introduction in commercial ICP-MS instrument. These systems involve the random arrival of polydisperse aerosol droplets into the plasma. But, monodisperse aerosol droplet generators have been used for the NP suspension in single-particle mode (Franze et al. 2012; Gschwind et al. 2011, 2013; Borovinskaya et al. 2013). These generators can produce pulsed microdroplets as well as droplet size below 100 micrometers. So, these generators can be coupled to existing delivery systems for transport of the droplet to plasma. By using these systems, nebulizing efficiency can be increased more than 95% and size/mass calibration can be performed just by using dissolved standards and knowing the diameter of the droplets (Gschwind et al. 2011, 2013).

The main limitation of this technique of spICP-MS is the lower size limit of particle detection, which is generally between 20 and 30 nm. Hence, particles below 20 nm cannot be detected easily (Laborda et al. 2011; Pace et al. 2012). Another demerit is that real-world samples must be altered so as to be compatible with common ICP-MS sample introduction system. While considerable progress has been made, spICP-MS is still best considered emerging techniques with many advancements yet to come. In facts, the technique is already being applied as both a research tool and a metrological tool.

2.9 Applications of spICP-MS

spICP-MS is an emergent technique which has many applications such as detection and quantification, sizing and size distribution, stability, dissolution and migration of NPs from solid matrices. This technique has been used for detection of NPs in environmental samples of wastewaters from treatments (Tuoriniemi et al. 2012). Mitrano et al. (2012) detected and quantified both dissolved and nanoparticulate Ag at ng/L level in influent and effluent samples from a wastewater treatment plant. The use of consumer products containing NPs can cause diseases after their release into the environment. Reed et al. (2013) have used the residual catalyst metals such as Co, Yt, Mo, and Ni contained in carbon tubes for their detection at ng/L levels.

The European Commission (EC) has defined the term “nanomaterial” for regulatory purposes based on the size of NPs and their number size distributions (European Commission 2011). The Institute for Reference Materials and Measurements of EC (Linsinger et al. 2012) has published a report related to the requirements on measurements for the implementation of this definition. Laborda et al. (2013) have applied this technique to estimate the number size distribution of different commercial NMs in context of the EC definition. Loeschner et al. (2013) have obtained the size distribution of AgNPs in meat digestates using ICP-MS in single-particle mode. Pace et al. (2012) have demonstrated that spICP-MS can be used and able to get size distribution of AgNPs across different sizes and number concentrations.

Under biological or environmental conditions, NPs can undergo a number of transformations. Some metal-containing NPs such as Zn and Ag can dissolve and release soluble species. spICP-MS has been used for transformation products both as dissolved and aggregates (Hadioui et al. 2013). Dissolved Ag from AgNPs under lab conditions have been quantified using this technique. Walczak et al. (2012) have studied the fate of AgNPs during gastrointestinal digesting by using spICP-MS as well as SEM and DLS. Releasing and dissolution from plastic food storage container have been studied using spICP-MS (Goetz et al. 2013; Echegoyen and Nerin 2013). This methodology and technique has not been exploited fully till now. It has potential to study migration and dissolution of NPs from solid matrices.

2.10 Recent Advances and Future Prospects

In the last few years, spICP-MS has developed from a niche technique to a powerful tool for the analysis of ENMs. As the development in spICP-MS techniques is taking place, additional instrument capabilities such as very short detector dwell times in the range of microseconds, automated data reduction software and hyphenation with front-end separation techniques (field-flow fractionation) are being viewed as necessary improvements by the instrument manufactures. Automated data processing softwares have been enveloped recently. These software packages automate many of the processes in single-particle analysis such as determining the transport efficiency, calculating their diameter of the analyzed particles, and creating histograms to display the size distribution. Other inputs such as dwell time, mass fraction and particle density are user-defined; hence, a wide breadth of NMs can be analyzed without the need to manually perform the data analysis. In addition to this, particle number concentration and dissolved analyte concentration are calculated automatically which further adds to the wealth of information obtained from spICP-MS analysis.

2.11 Conclusions

There are several studies addressing toxicological concern about NPs of different composition. In addition to size and shape, chemical composition is another important factor in determining toxicity of ENPs. Complete characterization of nanomaterials is important for interpreting the results of toxicological and human health studies. Although a number of techniques for characterization of ENPs are available, spICP-MS has proven to be a powerful technique for the detection and characterization of aqueous dispersion of metal-containing engineered nanomaterials. This technique is capable of rapidly providing information pertaining to size, size distribution, particle number concentration and major elemental composition with minimal sample perturbation due to the high-throughput single-particle counting technique and elemental specificity of ICP-MS.

The strengths of spICP-MS include no instrument modification and excellent detection capability in terms of nanoparticle number or mass concentration. However, demerits include measurement of only one or at most two isotopes in a single analysis. The detection power in terms of nanoparticle size is still somewhat lacking, with typical limits of detection ranging from 10 nm to 20 nm for monoisotopic NPs. Samples must be prepared or altered in such a way as to be compatible with ICP-MS sample introduction system. Advances in data acquisition and signal processing have resulted in a wider breadth of nanoparticle analysis. While considerable progress and much advancement have been made, the spICP-MS is still best considered an emerging technique in understanding the environmental, health and safety implications of inorganic engineered nanoparticles.

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Chapter 3
Green Synthesis of Microbial Nanoparticle: Approaches to Application

Jyotika Purohit, Anirudha Chattopadhyay, and Nirbhay K. Singh

3.1 Introduction

Nanotechnology is an emerging field blending the flavor of the scientific basis of physics, chemistry, and biology to exploit distinct technological advances for manipulating and creating tools or structure of individual molecules or their organized aggregates referred as nanoparticles (NPs). These nanoparticles (NPs) are the functional products of nanotechnology and possess the catalytic, optical, electronic, magnetic, and antimicrobial properties compared to the conventional polycrystalline materials. These NPs can be produced in various shapes such as spheres, triangles, hexagons, rods, wires, and tubes (Mandal et al. 2006) usually having dimensions of 100 nm or less (Huber 2005). Traditionally, they can be synthesized by a wide variety of physical and chemical methods such as UV irradiation, pyrolysis, lithography, laser ablation, ultrasonic fields, chemical vapor deposition, photoinduced reduction, aerosol technologies, and microemulsion synthesis techniques which are employed for the production of various metal NPs such as gold, silver, platinum, cadmium, iron, and palladium (Dhillon et al. 2012). These approaches end up with several limitations in terms of high energy, temperature, pH, and pressure requirement with huge monetary expenses. Moreover, the use of hazardous substances such as organic solvents, reducing agents, and stabilizers and the release of toxic by-products enhance the toxicity problems. Hence, the contamination of toxic agents with the synthesized NPs has prevented their clinical, agricultural, and other biological and environmental application

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(Prasad et al. 2014). In order to address these limitations, green synthesis came into existence recently to restrain the ecosystem through available naturally biodegradable resources for its biosynthesis (Gibney 2015; Malik et al. 2014).

Green synthesis of NPs has gained the momentum over the last few years due to their distinctive properties, wider applicability, and environmental sustainability. The development of NPs through green chemistry is an eco-friendly technique (Rai et al. 2012; Prasad 2014; Prasad et al. 2018a). The superiority of green NPs over their chemical counterpart lies on their organic origin and non-toxic property. Besides this, green synthesis involves a wide range of environmentally acceptable methodology with low-cost production in lesser time (Prasad et al. 2017). These green NPs are synthesized using diverse biological resources available in nature such as plants (Pantidos and Horsfall 2014), algae (Castro et al. 2013; Aziz et al. 2014, 2015), actinomycetes (Singh et al. 2016), bacteria (Li et al. 2011; Vijayaraghavan and Kamala Nalini 2010; Prasad et al. 2016), yeast (Ingale and Chaudhari 2013; Menon et al. 2017), fungi (Dhillon et al. 2012; Moghaddam et al. 2015; Prasad 2016, 2017; Prasad et al. 2018b), and viruses (Merzlyak and Lee 2006; Shah et al. 2015), or their secondary metabolites and by-products. The natural ability of these organisms and possibility to utilize them for synthesizing inorganic materials on a nano- and microscale served as driving fuel for the development of a relatively new and unexplored area of research. Feasibility of biosynthesis of "green" NPs primarily lies on the choice of appropriate medium (Li et al. 2011). Among many organisms, plant extracts are extensively exploited as the good media and are suitable for large-scale biosynthesis. But stability of plant-originated NPs differs significantly with the variation in biochemical composition of plant extracts of the same species (Duran et al. 2011). To overcome this problem, synthesizing NPs using microbial resource is relatively advantageous as microbe-secreted proteins confer better stabilization of NPs (Selvakumar et al. 2014). Furthermore, huge structural diversity and easy culturability of microbes ensure their innate property for synthesis of green nanoparticle (NP) which could be regarded as potential biofactories for NP synthesis.

Exploitation of microbial resource for green synthesis of NPs is considered an excellent strategy. So far, wealth of information is available on microbial nanotechnology, but very little information highlights their substantial mechanism of biosynthesis and stabilization along with their potential applications. In this article, we try to incorporate all possible aspects of NP synthesis procedures with aim of long-term stabilization and wider application in biomedical sciences, biosensors, chemical sensing, antimicrobial, drug delivery, mechanics, magnetic, energy science, agricultural, environmental, and other industrial sectors (Prasad et al. 2016). This will not only provide an overview of green synthesis of NPs using various microbes as a biotemplates but also would focus to find out possible approaches for minimizing the negative impacts of synthetic procedures and their accompanying chemicals and derivative compounds and ultimately to harness their sustainability in long run.

3.2 Nanoparticles and Their Green Sources

The term “nano” has been derived from its Greek origin “nanos” meaning dwarf. Usually the particles or molecules of any shape having a dimension in the range of 1×10^{-9} to 1×10^{-7} m are called nanoparticles (Banik and Sharma 2011). A NP is a collection of about tens to thousands of atoms measuring about 1–100 nanometers in diameter, created by atom aggregation to form amorphous, crystalline, or semicrystalline, zero-dimensional ($0D^o$) nano structure (with no dimensions longer than 100 nm). The essential criteria to be a NP is its size in nanoscale and structural arrangement, ability to work at the atomic or molecular level, and its novel, significantly changed, and useful physical, chemical, and biological properties not previously created or observed (Cao 2004). These may be available in the form of nanopowder or nanocluster or nanocrystal. These nanomolecule or nanoparticles are utilized for the creation of materials having dimension beyond the nanometer scale (>100 nm) and fundamentally new molecular organizations and functional characteristics. Other than changes in physical, chemical, and biological properties, two principal factors, i.e., the ratio of surface area to volume and the size of the particle, become the main determinant (Mazhar et al. 2017). Usually, NPs with lower particle size have a much greater surface area as compared to larger particles; and as the particle size decreases, dominance of the surface atoms increases over the interior of the particle. This results in the enhancement in catalytic activity of NPs, both in isolation and its interaction with other materials that helps to form nanocomposites, having special properties such as increased strength and chemical resistance (Mohanpuria et al. 2008; Prasad et al. 2017). Thus, size and shape of the nanomaterials can be controlled experimentally to generate *one-dimensional, two-dimensional, or three-dimensional nanostructures like* nanotubes, nanorods, nanowires, nanofilms, nanolayers, nanocoatings, etc.

NPs are generally available in organic or inorganic form. Of them, metallic NPs are the most common. They can be synthesized physiochemically, biologically, or through a hybrid of these techniques (Mazhar et al. 2017). Various physical methods, such as vapor deposition, lithography, mechanical attrition, laser ablation, laser pyrolysis, and ion sputtering, have been utilized, but these are not very popular, and the yield is very less (Li et al. 1999; Narayanan and Sakthivel 2010; Panigrahi et al. 2004). However, NP synthesis by chemical process is the traditionally most exploited approach. Their chemical synthesis involves the conversion of metallic ions to NPs with the help of various reducing agents, such as sodium borohydride, potassium bitartrate, methoxypolyethylene glycol, hydrazine, etc., via several methods like hydrothermal, conventional heating, anodization, deposition precipitation, wet oxidation, electrodeposition, and sonication (Kim et al. 2007; Li et al. 1999; Mallick et al. 2004; Panigrahi et al. 2004; Tan et al. 2003). However, these methods are usually highly expensive and very labor-intensive. Moreover, the use of some toxic chemicals and generation of hazardous by-products raise the environmental safety issue and health concerns to living organisms. Therefore, their application is restricted in biological, medical, and agricultural sectors (Prasad et al. 2017; Tarafdar et al. 2013).

To overcome such issues, green synthesis approach opens up new window to develop low-cost, non-toxic, biocompatible, and environmentally safe metallic NPs that have wider application in various sectors. A vast natural source of biological entities including plants, plant extracts, algae, fungi, yeast, diatoms, bacteria, actinomycetes, and viruses have been exploited in the development of metallic NPs (Li et al. 2011; Prasad et al. 2016; Thakkar et al. 2010). Due to their rich diversity and the innate potential for synthesizing different kinds of NPs, they could be regarded as potential biotemplate for NP synthesis. This green approach gains importance due to non-requirement of high pressure, energy, pH, temperature, and toxic compounds that ensure added advantage over chemical and physical methods. In addition, the size and shape of the NPs can be controlled by manipulating the growth and other cellular activities of the template organism (Gericke and Pinches 2006; Mazhar et al. 2017). Furthermore, their secondary metabolites like amino acids, peptides and organic acids are also very efficient for biological formulation of various nanostructures.

Comparison of different biological entities is very crucial to determine their potential and utility for efficient synthesis of NPs. So far, plants are most commonly utilized as a cost-effective resource for the metal NP production with the advantage of faster rate of biosynthesis than other biological entities. Besides, lack of complexity in preparation and maintenance of bioculture ensure its suitability for large-scale production (Makarov et al. 2014; Shah et al. 2015). However, variation in biochemical composition of the plant extract creates problem in stabilization, structural variation, and catalytic activity of metal NPs.

Thus focus is now shifted toward the utilization of microbial resource for synthesis of NPs. Horizon of this spread from simple prokaryotes to complex eukaryotic organisms like bacteria, fungi, yeasts, actinomycetes, algae, and viruses, as they can be easily harvested for the fabrication of NPs due to their natural abundance and ease in culturing (Prasad et al. 2016). Their metal biodegradation potential can also be exploited for this purpose. These microorganisms have the ability to detoxify heavy metals that are present in their environment and turn them into their elemental form via oxidation and reduction reactions (Labrenz et al. 2000; Mehra and Winge 1991; Stephen and Maenaughton 1999). This process is performed either intra- or extracellularly through enzymatic action. The extracellular enzymes of microbial origin are capable of reducing heavy metals present in the surrounding of cell, whereas intracellular enzymes target only the metals (ions) that are transported inside the microbial cell (Li et al. 2011). The particles generated in these ways have higher catalytic activity with greater surface area like their microbial origin; these particles can sustain various environmental conditions of varying temperature, pH, and pressure (Shah et al. 2015). Thus, microbe-originated NPs can be very efficient for diverse fields of application (Zhang et al. 2011). Furthermore, these NPs can be designed in various shapes and size in comparison with other sources. Due to their non-hazardous nature and an improved efficiency, microbial resource is considered as one of the best resource for synthesis of metal nanoparticles.

3.3 Microbes as Biotemplates for Nanoparticle Synthesis

Biosynthesis of various NPs using microbial template is already well established. This microbial approach is evolved as an efficient system for synthesis of NPs due to their distinctive properties. Most importantly, these microbiological systems have huge structural variation from micro- to macroscale length that may be exploited as suitable biotemplate (Table 3.1, Fig. 3.1). Besides various microbial structures, their cellular components sized in nanoscale are also preferred. The biochemical constituents of the whole cell and its parts or cell extracts like exo-polysaccharides, proteins, and enzymes are involved in the catalysis of NP synthesis pathway, and hence, understanding their significance and mechanism in NP synthesis is crucial.

3.3.1 Bacteria as Biotemplate

3.3.1.1 Bacterial Whole-Cell Template

Various bacterial cells, possessing different morphological features and surface structures can be used as a biotemplate for nanoparticle synthesis. The composition of cell surface structure including cell wall with exo-polysaccharides or surface layer proteins is the key determinant. Basically sugars, proteins, and enzymes present in the microbial cell wall have different functional groups like carboxyl, phosphate, and amide groups that favor the metal binding and nucleation process for the aggregation and formation of NPs or nanostructures (Selvakumar et al. 2014; Iravani 2014). One such component is bacterial exo-polysaccharide that is used as a potential biotemplate for the metal NP synthesis. The polyanionic functional groups (i.e., hydroxyl, carboxyl, and amino groups) of the exo-polysaccharide layer form an interface with metal cations and speed up their reduction process. This exo-polysaccharide is also used as the efficient capping agents for stabilizing the primary structures of metal NPs through chelation (Gomaa 2017; Sanyasi et al. 2016; Sathiyanarayanan et al. 2017), thus controlling the particle size and shape. The mucoadhesion property of exo-polysaccharides leads to a neutral, low surface energy in NPs, thus preventing their agglomeration and ensuring uniform particle dispersion after long-term storage.

Similar to exo-polysaccharides, the bacteria cell wall also contains surface layer proteins (S layer) that form self-assembled nanostructures on its surface to maintain the cell shape. This S layer possesses large surface area with uniform pore size and interacts weakly with metal ions. This induces specific binding of molecules on S-layer lattices via covalent and non-covalent bond which can be used for the synthesis of metal NPs like gold (Au), cadmium (Cd), etc. (Selvakumar et al. 2014). After isolation, S layer protein is re-crystallized into defined symmetries on the solid support matrices like electron microscope grids, silica, glass, mica, liposome, carbon, or polymers. Usually modification in self-assembling of S layer can be

Table 3.1 Potential microbial biotemplates used for the biosynthesis of metal nanoparticles

Sr. No.	Microbial Source	Production site	Size (nm)	Type	Application	References
Fungi	*Fusarium oxysporum*	Extracellular	5–15	Ag	–	Ahmad et al. (2003a)
	Aspergillus niger	Extracellular	20	Ag	Antibacterial activity	Gade et al. (2008)
	Fusarium solani	Extracellular	5–35	Ag	Antimicrobial agent	Ingle et al. (2009)
	Verticillium luteoalbum	Intracellular	<10–100	Au	–	Gericke and Pinches (2006)
	Fusarium oxysporum	Extracellular	3–11	Zr	Space application as quantum dots	Bansal et al. (2004)
	Trichoderma viride	Extracellular	10–40	Ag	Antibacterial activity	Fayaz et al. (2009)
	Aspergillus fumigates	Extracellular	1.2–6.8	ZnO	Industrial, medical and agricultural sectors	Raliya and Tarafdar (2013)
	Aspergillus tubingensis	Extracellular	28.2	$Ca_3P_2O_8$	Agricultural, biomedical and engineering sectors	Tarafdar et al. (2012)
	Rhizopus oryzae	Cell surface	10	Au	Agricultural pesticides	Das et al. (2009)
	Rhizopus stolonifer	Cell surface	1–5	Au	–	Sarkar et al. (2012)
	Penicillium fellutanum	Extracellular	5–25	Ag	–	Kathiresan et al. (2009)
	Coriolus versicolor	Intracellular & Extracellular	25–75, 444–491	Ag	–	Sanghi and Verma (2009)
	Phaenerochaete chrysosporium	Extracellular	50–200	Ag	–	Vigneshwaran et al. (2006)
	Phoma sp. 3.2883	Extracellular	70–75	Ag	–	Chen et al. (2003)
	Fusarium oxysporum	Extracellular	5–15	TiO_2, SiO_2	Cosmetics	Bansal et al. (2005)

Bacteria	*Pseudomonas aeruginosa*	Extracellular	15–30	Au	Bioremediation	Husseiney et al. (2007)
	Bacillus subtilis	Intracellular and extracellular	5–10	Ag & Au	–	Reddy et al. (2010)
	Escherichia coli	Intracellular	2–5	CdS	Biosensors	Kowshik et al. (2002a)
	Clostridium thermoaceticum	Intracellular and extracellular	2–5	CdS	–	Velusamy et al. (2016)
	Bacillus cereus		20–40	Ag	Antibacterial activity	Sunkar and Nachiyar (2012), Iravani (2014)
	Pseudomonas stutzeri AG259	Intracellular	35–46/200	Ag	–	Haefeli et al. (1984)
	Rhodobacter sphaeroides	Extracellular	10.5	ZnS	–	Bai and Zhang (2009)
	Streptomyces albidoflavus	Intracellular	10–14	Ag	–	Velusamy et al. (2016)
	Klebsiella pneumoniae	Extracellular	5–32	Ag	–	Velusamy et al. (2016)
Yeast	*Candida glabrata*	Intracellular and extracellular	20–29	CdS	Physiological	Dameron et al. (1989)
	Schizosaccharomyces pombe	Intracellular	1–1.5	CdS	–	Kowshik et al. (2002a)
	Pichia jadinii	Intracellular	100	Au	–	Mariekie and Anthony (2006)
	Yarrowia lipolytica NCIM3589	Cell surface	variable	Au	–	Pimprikar et al. (2009)
	Yeast sp.	Extracellular	10–80, 80–200	$Zn_3(PO4)_2$	Electronics	Yan et al. (2009)
	Saccharomycetes. cerevisiae	Intracellular	3–10	Sb_2O_3	–	Jha et al. (2009)
	Torulopsis sp.	Intracellular	2–5	PbS	–	Kowshik et al. (2002b)

(continued)

Table 3.1 (continued)

Sr. No.	Microbial Source	Production site	Size (nm)	Type	Application	References
Algae	*Chlorella vulgaris*	Extracellular	9–20	Au	Antitumor	Luangpipat et al. (2011)
	Scenedesmus sp.	Extracellular	15–20	Ag	–	Velusamy et al. (2016)
	Spirulina platensis	Extracellular	–	Au, Ag	Bioaccumulation	Chakraborty et al. (2009)
	Sargassum wightii	Extracellular	–	Au, Ag		Singaravelu et al. (2007)
Virus	Tobacco mosaic virus (TMV	Intracellular and extracellular	45–80	SiO_2 , CdS, PbS, Fe_2O_3	–	Shenton et al. (1999)
	M13 bacteriophage	Intracellular and extracellular	50–100	ZnS and CdS	–	Mao et al. (2003)
	Tobacco mosaic virus (TMV)	Extracellular	Variable	Silica	–	Royston et al. 2009)
Actinomycetes	*Thermomonospora* sp.	Extracellular	8	Au	–	Ahmad et al. (2003b)
	Rhodococcus sp.	Intracellular	5–15	Au	–	Singh et al. (2016)

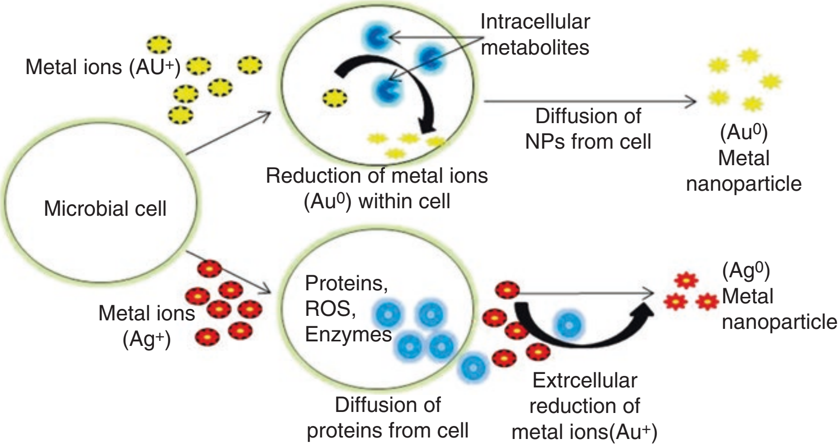

Fig. 3.1 Mechanism of nanoparticle synthesis by microbes

possible depending upon the metal NPs to be synthesized. S layer-coated grids act as the biotemplate and are dipped in a solution containing metal salt for coating and synthesis of NPs of defined shape and size (Iravani 2014).

3.3.1.2 Bacterial Cell Appendages

Other than the whole bacterial cell, appendages like flagella and pili are also used as the biotemplate for the synthesis of NPs. These flagella and pili are self-assembled filamentous nanostructure made of proteins, like flagellin and pilin, respectively. Their self-assembling property is explored and exploited for the fabrication of nanostructured materials with precise shape and size (Sleytr et al. 1993).

Bacterial flagella have unique mechanical properties like stiffness and elasticity that ensure durability and stability to withstand very high temperature and extreme pH and adaptability to the changing environment through helical rearrangement of flagellin. This property of flagellin protein can be used for de-polymerization into monomers followed by re-polymerization into modulated flagellar filaments of desirable dimensions and structures (Kowshik et al. 2002a, b). During re-polymerization, reshuffling of amino acids leads to different sequence and nature of polymers. These polymerized flagella can be used as the biotemplate for the synthesis of NPs with superior electrical conductivity (Gopinathan et al. 2013; Velusamy et al. 2016). Further, the flagellin protein subunits can be modified genetically to enhance their affinity for metal ions and fabrication of hybridized metal nanotubes.

Bacterial pili are a small hair-like structure with diverse functional category. One such functional category of pili promotes interaction with inorganic electron acceptors and is involved in extracellular electron transfer. Such types of electrically conductive pili (e-pili) act as microbial electrode, called “biological nanowires,” and are found in *Geobacter* sp. and *Shewanella* sp. This property of pili can be exploited for the biosynthesis of different nanostructures. Deposition of NPs like titanium dioxide (TiO_2), zinc oxide (ZnO), and aluminum oxide (Al_2O_3) on these pili forms nanofibers that enhance the extracellular electron transfer. These oxide-coated nanostructures are also applicable in bioelectric production (Selvakumar et al. 2014).

3.3.2 Fungi as Biotemplate

Like bacterial cell, fungi are also a potential candidate for the synthesis of metal NPs. The enzymes and proteins synthesized by fungi help in reducing the metal salts to NPs; therefore, they are invariably used for the synthesis of metal NPs. Usually fabrication of metal NPs of varying size and shape occurs either extracellularly or intracellularly (Sastry et al. 2003; Abdel-Aziz et al. 2018). Extracellular synthesis occurs through biosorption of metal ions on the cell wall of fungal filament and reduction by secretion of extracellular enzymes bacterial cell and metabolites, whereas intracellular synthesis involves influx of metal salts within the fungal cell followed by bioreduction into metal ions (Dhillon et al. 2014; Pantidos and Horsfall 2014). Usually, fungi are preferred over bacteria for large-scale production of NPs. Their versatility in shapes (from unicellular structures to microfilaments), wider adaptability in distinct ecology, and tolerance to environmental stress favor them for the purpose (Velusamy et al. 2016). Furthermore, fungal mycelia have large surface area for binding with metal salts and grow more biomass which results into rapid synthesis of metal NPs. Hence, exploration and exploitation of fungi for the fabrication of NPs become more advantageous (Prasad 2016, 2017; Aziz et al. 2016, 2019).

3.3.3 Algae as Biotemplate

Synthesis of NPs using algal biotemplate is another unique approach. The use of algal species in fabrication of metal NPs is totally subjected to their structural/physical features and secreted biomolecules (Davis et al. 2003; Siddiqi and Husen 2016). Diverse biomolecules like sugars, proteins and secondary metabolites secreted by various algae play crucial role in the biogenesis of NPs. Since algal membrane proteins are important for templating metals ions, extracellular polysaccharides help in the reduction of different metal ions and stabilization of metal NPs. Secondary metabolites (flavonoids and terpenoids) are effective for capping and stabilizing the metal nanoparticles; thus, altogether the design and size and shape of metal NPs (Anwar 2018; Kannan et al. 2013; Sharma et al. 2015;

Vasquez et al. 2016). Furthermore, existence of skeletal and morphological variability within algal species supports the production of nanostructures. NPs of different dimensions and morphology (spherical, elongated or irregular) are obtained depending on algal species (Patel et al. 2015). Algae are supposed to be advantageous for nanoparticle synthesis because it favors cost-effective and large-scale synthesis of highly stable, safe, and non-toxic nanoparticles with better biological properties (Aziz et al. 2014, 2015).

3.3.4 Virus as Biotemplate

Virus is a nucleoprotein particle containing genomic nucleic acid surrounded by proteinaceous capsid. Due to their unique sizes in nanoscale and shapes, they are considered natural nanoarchitectures. The three-dimensional (3D^{o}) structure of viral particles is densely covered by protein subunits, which in turn is made up of amino acid with carboxylate, amino, and thiol group side chains that represent a highly reactive layer and have high affinity for interacting with metal ions, and thus helps in their nucleation and metallization at the external surface (Flenniken et al. 2009; Shah et al. 2015; Selvakumar et al. 2014). Further, virus particles have a hollow inner cavity inside the viral capsid, where some metals can diffuse and interact with internally projecting amino acid side chains (Pokorski and Steinmetz 2011; Wen et al. 2012). Thus, inner cavity of viral particles can serve as a nanoreactor for fabrication of metal nanomaterials of desired sizes and shapes. Hence, external surface of coat protein and its interior cavity can be exploited for the designing of various metal nanowares, nanorods and nanotubes.

3.4 Approaches for Microbes-Derived Nanoparticle Synthesis

Synthesis of NPs can be achieved via two basic approaches, viz., top-down approach and bottom-up approach (Iqbal et al. 2012). In the top-down approach, a large bulk material is fragmented into fine particles by size reduction using lithographic techniques (grinding, ball milling, chemical etching, sputtering, thermal/laser ablation, etc.). This leads to the severe plastic deformation into nanoscale. It is a costly, very slow, and time-consuming approach and, thus, not suitable for large-scale production. However, in the bottom-up approach, NPs are synthesized through self-assembling of atoms or molecules into new nuclei that build up to NPs or nanostructures (Mazhar et al. 2017). Fabrication of NPs through this approach is more advantageous because of more homogeneous and less defective production of nanostructures with minimum cost. The aggregation of atoms or ions to produce NPs can be induced using chemical and biological methods. Among chemical methods, chemical precipitation, vapor deposition, sol-gel process, and pyrolysis are mostly applicable. These methods involve the chemical reduction of ions using different organic and inorganic reducing agents, such as ascorbic acid, citrate, glucose, dextrose, ethylene glycol, hydrazine, paraffin, sodium borohydride ($NaBH_4$), etc. (Ahmed et al. 2016; Iravani et al. 2014). This is followed by the use of capping agents for the stabilization of synthesized NPs. Although chemical synthesis of NPs is well established and very common, this approach is ecologically unfriendly and a highly expensive one. Thus, biological synthesis of "green" NPs is favored using diverse biological entities like microorganisms, plant extract, plant biomass, etc. (Bhattacharya and Gupta 2005; Dhillon et al. 2012). This green synthesis of NPs follows the bottom-up strategy where the reduction of atoms or ions occurs by biological reducing agents like polysaccharides, proteins, enzymes or metabolites of microbial origin. This is followed by nucleation of reduced metal atoms for subsequent growth and formation of nanostructures using microbial structures and other biological substances as the biotemplate. The advantage of green synthesis includes feasibility of designing various nanostructures like nanorods, nanosheets, etc. with more homogeneous composition and stability due to reduction of Gibb's free energy (Thanh et al. 2014; Shah et al. 2015). Thus green synthesis of NPs is progressing as a key approach for the production of NPs and could be preferred over chemical and physical methods due to its inexpensive, energy-saving, and eco-friendly nature (Reddy et al. 2012).

Biosynthesis of microbial NPs can be done using intracellular and extracellular synthetic methods. For intracellular synthesis, the microbial cell culture is employed with optimized metal salt concentration and incubated at desirable temperature and pH for production of NPs, whereas for extracellular synthesis, the crude extract of microbial cell culture (free from microbial cells) is mixed with metal salt solution and incubated for the reduction and stabilization of NPs (Fig. 3.2) (Baker et al. 2013; Velusamy et al. 2016). To synthesize NPs of precise size and shape, various

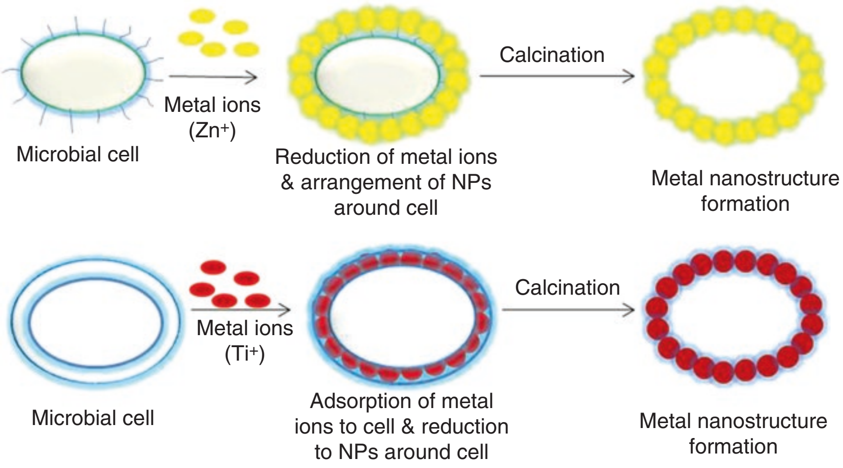

Fig. 3.2 Process of nanostructure formation by microbial templates

physiological parameters such as microbial source, nutrient composition, pH, temperature, metal salts concentration, etc. have to be optimized for specific incubation period and condition (static or shaking) (Baker et al. 2013). To get desirable NPs, selection of microbial agents is done based on their growth rate and ability to produce diverse biomolecules like enzymes, proteins, and metabolites that are essential for reducing metal ions. For optimization of bioreduction process, harvesting time of microbial cells or their biomolecules is also an important factor as the protein function and enzyme kinetics are variable at different courses of time for incubation (Khandel and Shahi 2016; Prasad et al. 2017). Thus optimization of all the physiological parameters is necessary for production of NPs with precise size, shape, chemical composition, etc**.**

3.5 Application of Microbial Nanoparticle

Nanoparticles of microbial origin have numerous potential applications in different fields such as cosmetics, coatings, packaging, electronics, agriculture, food and beverages, bioremediation, diagnostics, and biomedicines. Some of these applications are discussed in this section.

3.5.1 Cosmeceutical Industry

Novel nanocarriers like liposomes, nanoemulsions, solid lipid NPs, nanostructured lipid carrier, nanocapsules, etc. are used as cosmeceuticals to condition the skin, hair, and nail and for lip care, aging, and hyperpigmentation due to their biocompatible, self-cleansing, skin-compatible, antimicrobial, and dermatological behavior (Singh et al. 2016). Generally, NPs possess a wavelength below the critical wavelength of light which renders them transparent. This property makes them suitable for application in cosmeceutical industry (Raj et al. 2012). Metal NPs, viz., zinc (Zn) and titanium (Ti) oxide NPs, have been used in sunscreens, as they are transparent to visible light as well as absorb and reflect UV rays encountered in their way to be effective for skin conditioning (Pierfrancesco 2010). These properties of nanoscale materials in cosmetics impart a widespread use in personal care industries.

3.5.2 Electronics and Catalysis

Day by day, increasing importance of less power-consuming, large-sized, highly bright display technology encourages the use of nanoparticles. Nanocrystalline lead telluride, cadmium sulfide, titanium dioxide, zinc selenide, and zinc sulfide are mostly used in the light-emitting diodes (LED)-based modern displays system in computer monitors and television (Ealias and Saravanakumar 2017). Further, development of portable electronic gadgets such as mobile phones, laptops, and computers led to the extensive demand for compact, lightweight, and high-capacity batteries. In such batteries, considerably high energy can be stored compared to traditional batteries due to their foam-like (aerogel) structure. NPs are the ideal choice for separator plates in batteries made from nanocrystalline nickel, palladium, carbon-coated silicon nanowires, and metal hydrides, due to their large surface area requiring increased energy densities and thus decreased recharge times and long-lasting storage density (Lu et al. 2010).

On the contrary, microorganisms secrete large amounts of enzymes, involved in the enzymatic reduction of metals ions to produce metal NPs, which have characteristic features similar to chemically synthesized nanoparticles (Palomo and Filice 2016). Extremely large surface area-to-volume ratio of these nanoparticles functions as efficient catalyst for the production of chemicals. So, they have been widely used to improve various reactions as catalysts (Crooks et al. 2001). It is exemplified in AuNPs which are biosynthesized by using cell-free extract of *Trichoderma* sp. (at 30 °C) for producing nanoparticles of size 20–30 nm (Li et al. 2004; Mishra et al. 2014) and unique, anisotropic planar shapes that are applied in photonics and optoelectronics. The reduction of AuNPs through different solvents enhances their utility as future chemical sensors for reducing cost and improving performance significantly.

3.5.3 Medicine and Health

Deployment of nanotechnology in the medical field is enriched by their application as nanomedicine in healthcare (Li et al. 2011). This application of nanobiomedicine is expanded in diverse aspects like biosensors (Mohanpuria et al. 2008), detoxification of biological fluids (Singh et al. 2016), drug and gene delivery agents (Li et al. 2011), detection and diagnosis of pathogens (Nath and Banerjee 2013), and treatment of human diseases via tissue designing (Gurunathan et al. 2009), DNA analysis (Razavi et al. 2015), tumor destruction via heating/hyperthermia (Shinkai et al. 1999), MRI contrast enhancement (Weissleder et al. 1990), and phagokinetic examinations (Parak et al. 2002). Of them, microbe-originated metallic nanoparticles have promising usage in drug delivery, formulation of antimicrobial agents, and development of biosensors.

3.5.3.1 Drug Delivery

Accurate positioning of drug in desired dosage to their target sites (in cells or tissue of choice) at right time is the main concern in designing and developing of novel drug delivery systems. Therefore, total drug consumption and its side effects can be reduced significantly to make it precise, safe drug delivery to achieve the maximum therapeutic impact (Dhillon et al. 2012). Since the last decade, NPs have been widely investigated as a carrier for drug delivery (Gref et al. 1994). Silver (Ag) nanoparticles have gained importance as drug conveyors which can easily reach at targeted place due to their tiny size through the blood-brain cellular barrier and the narrow epithelial joints. It also enhances pharmacokinetics and biodistribution of therapeutic factors and therefore reduces poison via their privileged gathering at the targeted position owing to their higher surface area-to-volume ratio (Moghaddam et al. 2015). Similarly, magnetic nanoparticles Fe_3O_4 (magnetite) and Fe_2O_3 (maghemite) have been dynamically investigated as they are known to be biocompatible for several purposes such as target cancer therapy (magnetic hyperthermia), stem cell sorting, gene therapy, trained drug delivery, DNA analysis, and MRI (Xiang et al. 2007). Additionally, metal NPs have been used in the spatial analysis of various biomolecules, including several metabolites, peptides, nucleic acids, lipids, fatty acids, glycosphingolipids, and drug molecules with higher sensitivity and spatial resolution (Li et al. 2011). The reproduction and repair of damaged tissue (tissue engineering) can be carried out with the help of metallic NPs. Among various metal NPs, AgNPs have been widely considered for surgical gloves and covers, antibacterial injury dressings, bed lines, and so forth; likewise, they have got various applications in the fields of indicative therapeutics.

3.5.3.2 Antimicrobial Agent

Nanoparticle synthesis using microbial template has been emphasized in recent years due to the huge abundance of microorganisms contributing resistance to multiple antibiotics. These biosynthesized nanoparticles can be utilized for many antimicrobial purposes, viz., antibacterial, antifungal, antiviral, and anti-inflammatory factors (Fayaz et al. 2010). Some inherent properties of nanoparticles such as a very high aspect ratio in the case of AgNPs allow them to easily interact with other particles and increase their antimicrobial efficiency manyfold (Thakkar et al. 2010). Fungi-mediated AgNPs showed powerful bactericidal potential against both Gram-positive and Gram-negative bacteria by anchoring and penetrating the bacterial cell wall and modulating the cellular signaling pathways through dephosphorylating putative key peptide substrates on tyrosine residues (Sadhasivam et al. 2010; Singh et al. 2008). Extracellularly synthesized silver nanoparticle or AuNPs using *Fusarium oxysporum* and *Trichoderma* sp. can be incorporated in several kinds of materials such as textile fabrics. These fabrics embedded with silver NPs possess antibacterial properties, are safe, and can be used in hospitals to prevent/minimize the infection of pathogenic bacteria such as *Staphylococcus aureus* (Duran et al. 2007). Owing to their important biomedicine properties, silver NPs produced intra- or extracellularly using living organisms could be of great value.

3.5.3.3 Biosensors

Nanoparticles retain electronic and optical properties which can be used in biosensor applications. Selenium (Se) nanomaterial crystals exhibit high surface-to-volume ratio, biocompatibility, and good adhering and electro-catalytic activity toward the reduction of H_2O_2. Therefore, they are particularly used for enhancing the production of settled materials for building HRP (horseradish peroxidase) biosensor. Thus, the Se nanomaterials-modified electrode will probably be promising for a wide range of applications related to the detection of H_2O_2 in food, clinical, pharmaceutical, industrial, and environmental analyses (Li et al. 2011). Similarly, AuNPs are utilized for various purposes, e.g., as labels for biosensors, for cure of hyperthermia, determination of glucose level in commercial glucose injections, and staining of biological tissues, and are being equipped for conveying vast estimation of biomolecules (Moghaddam et al. 2015). Therefore, NPs have been acted as a novel biosensor with high sensitivity providing non-hazardous intends to environmental quality and medication liberation.

3.5.4 Food Industry

At present, huge improvement in production, processing, packaging, and protection of food is achieved by incorporation of nanotechnology via increasing the shelf-life of different kinds of food materials and is also helpful in reduction of food wastage due to microbial infestation (Pradhan et al. 2015). Nowadays, nanocarriers are being utilized as delivery systems to hold food additives in food products without troubling their basic morphology. For example, a nanocomposite coating employed in a food packaging can directly introduce the antimicrobial substances and also provide a barrier from extreme thermal and mechanical shock on the coated film surface (Pinto et al. 2013). For example, an additive called nanodrops, is being used in the canola oil production industry, in order to transfer vitamins and minerals in the food (Sekhon 2014). Additionally, nanofiltration is a recent membrane filtration system for water purification widely used in food and dairy industries to remove solids, including bacteria and other parasites.

3.5.5 Construction

Nanotechnological application has also improvised the properties of cement-based materials for construction. Use of nanoscale materials in the preparation of cementing agents makes them quicker, inexpensive, and safer (Sanchez and Sobolev 2010). Nanosilica (SiO_2) or hematite (Fe_2O_3) nanoparticle is mixed with the normal concrete to improve its mechanical properties, strength, and durability (Shah et al. 2009). Similarly, the properties of steel, the most widely used material in the construction industry, can also be improved by using nanotechnological application. Use of nano-sized steel offers stronger steel cables for the construction of bridge. Another important construction material is glass, which can be developed to have self-cleaning, sterilizing, and antifouling properties using (TiO_2) nanoparticles for glass coat glazing that provides better blocking of light and heat penetrating through the windows (Sobolev et al. 2009). Hence, nano-SiO_2, Al_2O3, TiO_2, and quartz can be used in the re-engineering of materials to improve their functionality and wider use in construction industry.

3.5.6 Environmental Remediation

The unique physicochemical properties of NPs have made them an ideal choice to decontaminate air, water, and soil under environmental remediation since the second decade of the twentieth century (Dhillon et al. 2014). These NPs are reported to clean up the environment via removal of heavy metals, pesticides, herbicides, fertilizers, oil spills, toxic gases, industrial effluents, sewage, and organic compounds (Liu 2006). Importantly, the ability of microorganisms to utilize its inherent biochemical processes to transform inorganic metallic ions into metal NPs with their surrounding environment has led to a relatively new and largely unexplored area of research. Songara et al. (2018) tested the rate of transformation of photodegraded products of benzyl butyl phthalate (BBP), an environmental pollutant, by *Pseudomonas putida* and the photocatalytic ZnO nanoparticles and concluded that the photocatalytic activity of ZnO nanoparticles was dose- and time dependent in transformation of BBP. Studies have also shown that extracellularly and intracellularly synthesized green metallic NPs (Ag, Fe, Pt, Pt-Co, Pt-Ni, Fe-Co) are involved in detoxification of contaminants via redox reaction (Liu 2006; Shah et al. 2015). Furthermore, zero-valent iron (granular form of iron) has reducing and absorption property and is used in remediation of chlorinated compounds (Oh et al. 2016). Hence, it is a relatively recent development that material scientists have been keeping interest in such microorganisms that can become possible eco-friendly input in a number of environmental remediation technologies.

3.5.7 Agricultural Applications

It is noticeable globally that the agricultural sector is continuously hampered by different pests and pathogens which are adversely affecting plant growth and agricultural production, which results in high economic losses and poses a risk to the global food security (Ingale and Chaudhari 2013). To control these plant pests and diseases, farmers use various agrochemicals indiscriminately which results in deterioration of soil and water quality. The excessive and repeated use of these chemicals causes ecotoxicological effects and occasionally results in development of resistance against agrochemicals (Prasad et al. 2017). In some cases, these chemicals also enter in the food chain and get accumulated in the human body. More efforts should be devoted toward developing safe management methods that can replace synthetic pesticides with higher efficiency and also pose less danger to humans and animal health (Benelli and Lukehart 2017). Thus, green synthesis of metal nanoparticles using microbial template not only offers a novel, easy, environmentally safe, and cost-effective approach but also ensures sustainable pest management strategy. These nanopesticides play an important role in the recent development of non-toxic and promising pesticide delivery systems for increasing global food production in sustainable agriculture by reducing rate of application at least 10–15 times smaller than the applied classical formulation (Kah and Hofmann 2014). Alternatively, due to special properties like sensitivity and performance, these nanoparticles could be employed as biosensors in global positioning systems with satellite imaging of fields for distantly detection of crop pests, soil analysis and physiological stress such as drought (Fraceto et al. 2016). NPs exhibit very good transduction properties which are being explored for analytical purpose of agricultural products. In this regard, AuNPs have intrinsic properties and may be used as transducers for several improvements in agricultural products (Kandasamy and Prema 2015). Hence, nanoscale carrier can be utilized for the efficient delivery of fertilizers, pesticides, herbicides, plant growth regulators, etc. in smaller amount and have much better and prolonged management in the agriculture sector.

3.6 Advantages and Future Trends

Green synthesis of microbial nanoparticles has scaffolding advantages, including biocompatible, ecofriendly, and cost-effective production methodologies. Apart from that, there is no requirement of further stabilizing agents as microbial cell constituents itself act as capping and stabilizing agents (Kalishwaralal et al. 2009). However, the surfaces of microbial NPs gradually and selectively adsorb biomolecules when they come into contact with complex biological fluids, forming a corona that interacts with biological systems which provides additional efficacy over bare biological nanoparticles. This makes it a less time-consuming, high yielding, and valuable one-step process by reducing the number of steps required in physiochemical synthesis and by including the attachment of some functional groups to the nanoparticle surface to make them biologically active (Khandel and Shahi 2016). Some of the other advantages like synthesized nanoparticle size can also be controlled easily by various parameters like pH and temperature (Gurunathan et al. 2009). Nanoparticles with smaller curvature have greater catalytic activity; hence, angular shapes are preferable over spherical particles due to their smaller radii of curvature in same volume (Li et al. 2011). Sometimes, nanoparticles are coated with a lipid layer that confers physiological solubility and stability, which is critical for biomedical applications and is the bottleneck of other synthetic methods (Razavi et al. 2015).

There have been tremendous developments in the exploration of microbial biotemplates for green synthesis of nanoparticles and their applications in various sectors over the last decade. However, the research work is still in the early stage, and yet further exploration is needed to improve their synthesis, efficiency, and stability, keeping in view the variegated parameters like type of microorganisms, growth phase of microbial cells, growth mediums, synthesis conditions, pH, substrate concentrations, target nanoparticles source compound, temperature, reaction time, and addition of non-target ions, which might result into obtaining sufficient control of particle size and monodispersity. Moreover, research efforts should be carried out in manipulating cells at the genomic and proteomic levels with better understanding of the synthesis mechanism on a cellular and molecular level, including isolation and identification of the compounds responsible for the reduction of nanoparticles. Further breakthroughs are desirable in order to revolve the impression of nanoparticle technology into a rational practical approach.

3.7 Conclusion

Since origin of life on earth, microbial entities have developed and evolved in environment containing various inorganic materials and these microorganisms play important role in transformation of minerals from one form to another in nature. Moreover, sustenance of life on the earth require a large number of minerals. Plethora of literatures have been found on multidisciplinary approaches of inorganic nanoparticles synthesized by microorganisms through either intracellular or extracellular routes. Presently, these nanoparticles have become a prominent platform for a diverse range of biological applications in a reliable and greener way instead of chemical and physical methods involving toxic chemicals and high temperatures that are not only hazardous to the environment but are costly too. Moreover, the shift from physico-chemical methods to greener synthesis of nanoparticles is preferred because they are environmentally salubrious, sustainable, safe and ecofriendly. Numerous microbial groups have focused on alternative ways of synthesizing nanoparticles as described in this chapter from its approaches to applications. Still, the field of microbial biosynthesis of metallic nanoparticles is relatively new and underexplored; however, it shows great potential in development of newer technology as it provides a single-step process for biosynthesis of nanoparticles which attracts more researchers to go for future developments in the area of electrochemical sensor, biosensors, healthcare, pharmaceutical, environmental technology, and agriculture. Development of improved technology will surely open up several new and exciting possibilities in the use of bioprocessed nanoparticles in every sphere of life which can become a boon to the society.

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Chapter 4
Enzyme Nanoparticles: Microbial Source, Applications and Future Perspectives

Lekshmi K. Edison, V. M. Ragitha, and N. S. Pradeep

4.1 Introduction

Enzymes are considered as fundamental biological catalysts that have important function in biological regulations and biochemical reactions. The enzyme activity relies on its particular site known as active site, very specific for substrates where the substrates or reactants bind and convert them to products (Brahmachari et al. 2017). Enzymes are mostly proteins excluding catalytic DNA and RNA. Enzymes have an important role in several catabolic and anabolic processes; thereby, they are used for synthesis and biodegradation of molecules. Without enzymes, the biochemical reactions may not be possible in normal physiological conditions. One particular reaction is catalysed by a specific enzyme, and substrate specificity is based on amino acid sequence recognition in the catalytic site. In enzyme catalysis, the substrate is changed to a product after passing a high energy state. The enzyme lowers this energy and speeds up the process. The enzyme favours this energy transition by providing a specific environment (Mantsala and Niemi 2009).

Enzyme activity can be affected by different factors like temperature, pH, ionic state, substrate concentration, etc. The International Union of Biochemistry and Molecular Biology (IUBMB) recommends the optimum assay temperature is 30 °C. Enzymes are sensitive to pH and work efficiently in pH close to neutrality (Mantsala and Niemi 2009; Tischer and Wedekind 1999; Livage et al. 2001; DeSantis and Jones 1999). Ionic states also have significant role in substrate binding and catalysis. The substrate concentration is very much significant in rate determination because the lower substrate concentrations significantly increases the rate of

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enzyme-catalysed reactions. In addition to these parameters, incubation time also affects reaction rate and stability of the enzyme. But at higher concentrations, the reaction may be independent of substrate. Enzyme catalysis depends on the amino acids present in the active site in which the substrate binds to enzyme. The active site is a pocket or cleft-like structure, and its three-dimensional structure is obtained by the interaction and proper folding of the amino acids. Polar amino acids aspartate, glutamate, cysteine, histidine, lysine and serine are most probably frequently occupied in the active site. The active site is very flexible. In addition to the active site, some enzymes possess a prosthetic group, and they act like coenzymes. The activity of enzymes can be modified by considering these factors.

Enzymes have immense applications in many fields, which target to improve the life of humans. Enzyme purification and its large-scale production from multiple sources with versatile applications have been widely reported. Typically, enzymes from microbial source act as excellent catalysts and play superior roles in various industrial applications (Table 4.1). Medicine, beverage, textile, paper, leather, molecular techniques and biofuel production are some of the areas where the microbial enzyme has immense role. In the last century, many studies have been conducted about the isolation, production and characterization of microbial enzymes. Bacteria, fungi and yeast are the major sources of microbial enzymes (Pandey et al. 1999; Prasad et al. 2016). Natural enzymes from microbial source are readily accessible, very affordable, efficient and eco-friendly and require very less energy consumption (Edison et al. 2017). As a part of biotechnological developments, genetically engineered and modified microbial enzymes with enhanced activity have been readily available for industrial uses (Chirumamilla et al. 2001). Because of the huge application of microbial enzymes, studies still continue to modify its activity by the application of emerging technologies. Enzyme nanotechnology is one of such amazing fields which targets to increase the catalytic activity of enzymes using nanoparticles.

4.2 Nanoparticles and Nanotechnology

'Nano' is a prefix that indicates one 'billionth', or 10^{-9}, of something. Particles with 1–100 nm size range are defined as the nanoparticles (NPs) (https://pubs.acs.org/doi/pdfplus/10.1021/bk-2008-0996.ch001). This extremely small size helps to possess a large surface area than in the particle interior, and some exhibit quantum effects too. A great chemical and morphological diversity is associated with nanoparticles. Chemical diversity includes metals, metal oxides, polymers, semiconductors, carbon materials, organics, biological, etc., and morphological diversity in shapes includes cylinders, spheres, disks, hollow spheres, tubes, platelets, etc. These diversities help NPs to exhibit different properties, and they function in many different ways. They showed interesting optical properties such as absorption and/or emission wavelengths which depend on particle size and surface functionalization. The NPs can attain transparency when the size is critically below the wavelength of light. They can control the ionic potential, electron affinity and electron

Table 4.1 Industrially important microbial enzymes

Enzyme	Microbial source	Biotechnological use
Protease	*Bacillus* sp., *Aspergillus* sp.	Detergent industry
Pullulanase	*Klebsiella* sp.	Food industry
α-Amylase	*Bacillus* sp., *Aspergillus* sp.	Starch industry
β-Amylase	*Bacillus* sp.	Starch industry
Glucose isomerase	*Bacillus* sp.	Fructose syrup
Asparaginase	*E. coli*	Health industry
Penicillin amidase	*Bacillus* sp.	Pharmaceutical industry
β-Glucanase	*Bacillus* sp., *Streptomyces* sp.	Brewing industry, animal feed industry
Aminoacylase	*Aspergillus*	Pharmaceutical industry
Invertase	*Saccharomyces*	Confectionery
Pectinase	*Aspergillus*	Juice industry
Lipase	*Rhizopus, Candida*	Food industry, paper industry
Dextranase	*Penicillium*	Food industry
Glucoamylase	*Aspergillus*	Starch industry
Catalase	*Aspergillus*	Dairy industry
Cutinase	*Fusarium*	Detergent industry
Xylanase	*Aspergillus*	Baking industry
Lactase	*E. coli, Kluyveromyces* sp.	Dairy industry
Transglutaminase	*Streptoverticillium* sp., *Streptomyces* sp.	Baking industry
Cellulase	*Aspergillus, Trichoderma*	Brewing industry
Naringinase	*Aspergillus*	Brewing industry
Limoninase	*Aspergillus*	Brewing industry
Phytase	*Aspergillus*	Animal feed enzyme industry
Keratinase	*Aspergillus*	Leather industry
Ligninase	*Basidiomycetes*	Textile industry, bioremediation
Laccase	*Trametes hirsuta*	Polymer industry
Tyrosinase	*Trichoderma*	Polymer industry
Mannanase	*Bacillus* sp.	Detergent industry
Superoxide dismutase	*Corynebacterium glutamicum, Lactobacillus plantarum*	Cosmetics industry
Endoglycosidase	*Mucor hiemalis*	Cosmetics industry
Amidase	*Rhodococcus erythropolis*	Waste management
Nitrile hydratase	*Rhodococcus* sp.	Waste management
Oxygenase degradation	*Pseudomonas* sp., *Rhodococcus* sp.	Waste management

transport properties. The nanoparticle incorporated materials decrease the sintering and melting temperatures which provide better thermal conduction and magnetic behaviour. Hence, NPs are widely considered to have the potential to bring benefits in different areas. Thus, a new concept, nanotechnology, arose. This concept was introduced by physics Nobel laureate Richard P. Feynman (Feyman 1960). Nanotechnology concentrates on the production, manipulation and scaling up of nanoparticles (National Nanotechnology Initiative www.nano.gov) which have applications in biology, physics, chemistry, mathematics, material sciences and earth science. A new branch ‘nanobiotechnology’ has been forced to evolve from the huge diversified biological applications. This branch deals with the applications of nanotechnology in the field of biology which include fluorescent biological labels, drug and gene delivery, detection of proteins, enzyme modifications, probing of DNA, detection of pathogens, tissue engineering, tumour destruction, separation and purification of biological molecules and cells, phagokinetic studies, etc. (Salata 2004; Prasad et al. 2014, 2016, 2017). Among other fields, production and manipulation of enzyme nanoparticles are an emerging field of interest today because of their immense application in the field of medicine.

4.3 Enzyme Nanotechnology

Enzyme nanotechnology is an emerging field and targets to alter the catalytic activity of an enzyme by using nanoparticles. The major products resulting from enzyme nanotechnology are single enzyme nanoparticles (SENs) and enzyme nanoparticles (ENPs). Even though the terms somehow give idea about enzyme nanoparticles, but both are different in concept-wise. SENs are single enzymes which are surrounded by organic/inorganic nanomaterials, whereas ENPs are enzyme aggregates which are formed by attaching a number of enzymes on a nanoparticle. The methods of formation of these two enzyme nanoparticles are different.

4.3.1 Single Enzyme Nanoparticles (SEN)

The short catalytic time of enzymes reduces its efficacy in industries. Enzyme immobilization, enzyme modification, genetic modification and medium engineering are some of the approaches adopted by different groups to increase the stability of the enzymes (Tischer and Wedekind 1999; Livage et al. 2001; DeSantis and Jones 1999; Govardhan 1999; Mozhaev 1993). SENs overcome the issues associated with enzyme stability, and this technology was introduced in 2006 (Yan et al. 2006). The method itself creates a nanostructure, known as carrier which encloses the enzyme molecule inside it. The enzyme with the nanostructure is also known as caged enzymes.

4.3.1.1 Features of Single Enzyme Nanoparticles (SEN)

- Every SEN possesses only one enzyme.
- The SEN molecule is covered with a thin nanolayer.
- The nanolayer may or may not be continuous around an enzyme, but it should be porous enough to diffuse the substrate molecule to the enzyme.

4.3.1.2 Methods of SEN Synthesis

The process of synthesis of SEN is known as 'grafting from method'. Grafting from method can be categorized into macromonomer grafting and macroinitiator grafting. In macromonomer grafting, the enzyme is the macromonomer, and the carrier is built from the macromonomer (Sharma et al. 2005; Kumar et al. 2005; Madadlou et al. 2010). Here, the macromonomer does not have an initiator function for interacting the enzyme with the carrier, whereas in grafting from macroinitiator, the monomer acts as an initiator in the enzyme-carrier interactions (Lele et al. 2005; Heredia et al. 2005; Nicolas et al. 2006; Liu et al. 2007; Yan et al. 2007; Boyer et al. 2007; Ge et al. 2008; De et al. 2008).

The carrier material can be inorganic and organic (Fig. 4.1). The inorganic materials include mesoporous silica (Ma et al. 2004), superparamagnetic clusters (Hong et al. 2007; Yang et al. 2007), hollow metal (Kumar et al. 2005) and silica spheres (Sharma et al. 2005; Madadlau et al. 2010). The organic carriers include polymer gel (acrylamide-bisacrylamide spatial gel) (Yan et al. 2006), organic–inorganic hybrid polymer (Kim et al. 2006; Hegedüs and Nagy 2009a, b; Ge et al. 2011; Lin et al. 2012) and dendrons (Khosravi et al. 2012). The carrier molecule interacts with the enzyme through either covalent or electrostatic interaction. This carrier layer provides stability in the function of the enzyme. In addition to the type of material, the thickness of the carrier level also affects the activity of the enzyme. The layer should allow the diffusion of the substrate to the enzyme.

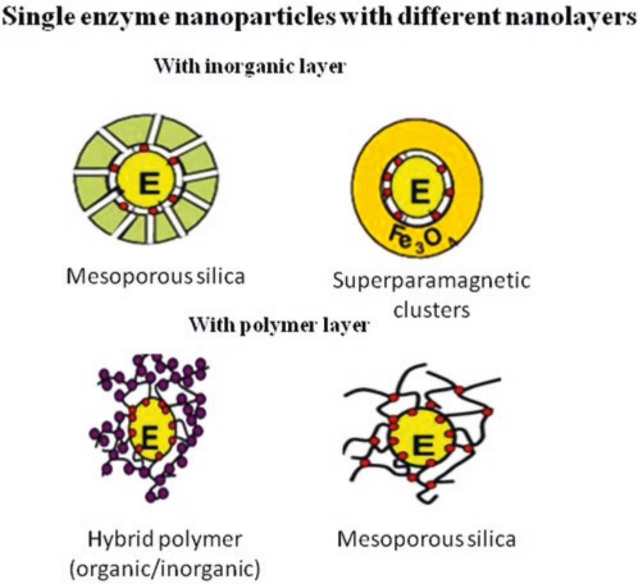

Fig. 4.1 Diagrammatic representation of SEN with nanolayers. (Reprinted with permission from Hegedüs et al. 2010)

The formation of SENs involves surface covalent modification, polymerization, hydrolysis, crosslinking and encapsulation (Fig. 4.2). The surface modification is done by using acryloyl chloride, which reacts with the amino groups of the enzyme. This reaction will provide vinyl groups on the surface of the enzyme (Kim and Grate 2003). This enzyme was dissolved in methane and treated with methacryloxypropyltrimethoxysilane (MAPS). MAPS is a vinyl monomer which possesses pendant trimethoxysilane groups. Vinyl polymerization was initiated with free radical, and the cold aqueous buffer was used to extract the product. In some, the process ends in the synthesis of SENs, but some people target to encapsulate the synthesized SENs into a gel to form single enzyme nanogels (Beloqui et al. 2017) or into porous silica to form single enzyme nanoporous silica (Kim et al. 2006).

Even though the production of SEN particles increases the stability of enzymes compared to free enzymes, the enzyme activity may lose due to the interaction of catalytic site with nanoparticles. SEN also prevents the mass transfer of the substrate to the enzyme due to the thickness of the carrier layer. The formation of single

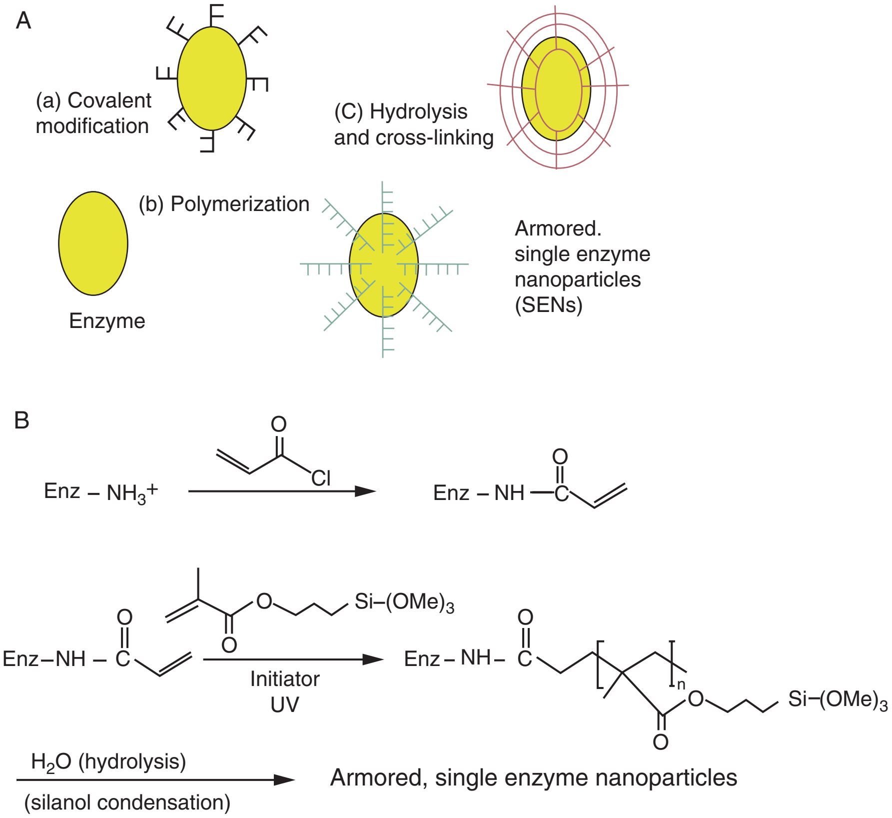

Fig. 4.2 Diagrammatic representation of SEN synthesis (**a**) and chemistry behind the synthesis. (Reprinted with permission from Kim and Grate (2003). Copyright © 2003 American Chemical Society)

enzyme nanoparticles increases the nanoparticle waste, which is difficult to degrade and causes the pollution. But the continuous efforts result in the generation of enzyme nanoparticles (ENPs) which can overcome the issues associated with SEN. The biocatalytic activity also increased compared to single enzyme nanoparticles.

4.3.2 Enzyme Nanoparticles (ENPs)

The aggregate or assemblage of enzyme molecules in a static protein configuration system of 10–100 nm planes is called enzyme nanoparticles. They have optical, electrical, thermal, catalytic, chemical and mechanical properties. They also have increased surface area than that of SENs. Nanoparticles conjugated with enzymes enhance the performance of enzyme-based sensors. However, the nanoparticles directly attached with enzymes may denature the enzymes. To avoid this situation, in enzyme nanoparticles, enzymes organized themselves as clusters in a systematized crosslinked sequence. This is the basic strategy used in ENPs-based biosensors (Sharma et al. 2011). The enzyme nanoparticle was first prepared and characterized by Liu et al. (2005) with horseradish peroxidase (HRP). Later, preparation and use of ENPs of glucose oxidase (GOD), uricase and cholesterol oxidase (ChOx) nanoparticles were reported (Sharma et al. 2011; Chawla et al. 2013; Kundu et al. 2013; Chauhan et al. 2014). They are unlike SENs, which are single enzyme molecules bounded with a network of nanoscale macromolecules.

ENPs bound to immobilization matrix (substrate) via covalent binding, entrapment, crosslinking and adsorption. In covalent binding, the side chain of amino acids present in enzymes creates covalent bond, and it helps to connect with functional groups of ENPs and supporting medium (D'Souza 1999). This is mainly due to the reactions of various amino acids, viz. aspartic acid, histidine and arginine, with certain functional groups including indolyl, hydroxyl, imidazole, etc. As a part of this crosslinking, the covalent bonding between biocatalyst and organic or inorganic reagents makes ligands. In the process of entrapment, the ENPs' movement is constrained in porous substances like agarose, gelatin and agar. Adsorption mechanism of ENPs can be achieved by immobilizing it onto insoluble matrix by ionic, hydrogen bonding and Van der Waal interaction. It has an advantage to reuse ENPs several times without affecting the functionality. Commonly used immobilized materials are Au electrode, nitrocellulose membrane and Pt electrode (Sharma et al. 2011; Kundu et al. 2013; Chawla et al. 2013).

4.3.2.1 Methods of ENP Synthesis

The formation of ENPs has been done by (1) desolvation and crosslinking, (2) emulsification, (3) simple coacervation method and (4) crosslinking in water–oil emulsion.

Desolvation and Crosslinking

The desolvation method for nanoparticle preparation has been well illustrated and documented (Shutava et al. 2009). It is a process of replacing/removing water molecules from a macromolecule by non-solvent interaction (Rank and Baker 1997). The desolvation method includes dissolving the solid enzymes and carrier proteins in a specific reaction buffer. The addition of desolvating agent, i.e. absolute ethanol, into the soluble enzyme/protein mixture under continuous stirring removes water molecules in between ions and molecules and promotes the aggregation of enzyme/protein molecules into nanoparticles. The desolvation process reduces molecular distance between the enzyme molecules and helps in creating enzyme clusters. Addition of high concentration of glutaraldehyde as a crosslinker ensures the molecular crosslinking of ENPs. Finally, crosslinked ENPs can be separated from free enzyme molecules by ultra-sonication and centrifugation. Addition of cystamine dihydrochloride or cysteine helps in the functionalization of purified ENPs by introducing -NH_2 or SH groups on ENPs surface (Liu et al. 2005). The enzyme nanoparticle production by desolvation has been employed in few enzymes such as horseradish peroxidase (Liu et al. 2005), cholesterol oxidase (Chawla et al. 2013), glucose oxidase (Kundu et al. 2013), uricase (Chauhan et al. 2014), etc.

Emulsification Method

Aqueous enzyme/protein solution is converted into a homogeneous emulsion with plant oil using high-speed homogenizer, which in turn helps in creating high dispersion particles. The emulsion prepared using high volume of preheated oil and temperature over 120 °C prompts the evaporation of remaining water and generates nanoparticles by irretrievable aggregation of enzyme/protein molecules (Scheffel et al. 1972).

Simple Coacervation Method

The coacervation method is one of the commonly used techniques of microencapsulation. It has been applied for the preparation of BSA nanoparticles (Sailaja et al. 2011). In this technique, the macromolecule is separated into two immiscible liquid phases, viz. a dense coacervate phase and a dilute equilibrium phase. This method comprises four successive steps carried with continuous stirring: (1) active substance dispersion into a surface-active hydrocolloid solution; (2) precipitation of the hydrocolloid by lowering the hydrocolloid solubility; (3) induction of polymer–polymer complex by adding a second hydrocolloid; and (4) stabilization and hardening of induced microcapsules by the addition of a crosslinking agent, for example, glutaraldehyde, formaldehyde or transglutaminase.

Crosslinking in Water-in-Oil (w/o) Emulsion

The method includes the emulsification of protein or enzyme aqueous solution in oil using homogenization with high pressure. The water-in-oil (w/o) emulsion is formed when it is dispensed into preheated oil. This suspension in preheated oil maintained the temperature above 100 °C with continuous stirring for aggregating and denaturing the protein contents in aqueous solution and for evaporating the water molecules so that the proteinaceous nanoparticles are formed (Ezpeleta et al. 1999).

4.3.2.2 Characterization and Kinetics of ENPs

Various spectroscopy and microscopy methods are employed for the characterization of enzyme nanoparticles. For example, size and shape can be revealed by the use of transmission electron microscopy. Resulting peak formation in UV absorption spectroscopy can also be used for distinguishing the free and nanoparticle-conjugated enzymes (Sharma et al. 2011). ENPs exhibit enhanced stability because of greater surface area, but its kinetic properties have not been yet reported. Although the immobilization of ENPs on metals and nitrocellulose membrane helps the study of kinetic properties of ENPs, as similar to all other enzymes, the incubation time, temperature and pH are indispensable factors that affect the enzyme kinetics of ENPs. The immobilization and crosslinking create a stronger conformational stability of ENPs, thus providing a positive thermostable effect on the activity of ENPs when compared to free enzyme. It also proved that the increase in pH helps to increase the ENPs stability. Slight changes in pH and temperature may invariantly affect the characteristics of ENPs than single enzymes. Another major factor which affects the ENPs' activity is micronization of substrate (Wu et al. 2000). Immobilization helps the enzyme to reuse and make them stable. Here, the ENPs with different supporting plates help to design different enzymatic models which can control the enzymatic reactions more easily. It uses the whole usage of reactants in the medium and also provides a very pure product without mixing any other molecule. These features make the ENPs as a promising tool for industrial economic successes.

4.3.3 Microbial Enzymes' Interaction with Nanoparticles

Microbial enzymes immobilized on nanoparticles have shown substantial improvement in enzymatic performance, which intensely depends on types of enzymes, supporting materials and conditions of immobilization. Immobilized enzymes under optimized conditions on nanomaterials frequently exhibited four advantages when compared with free enzymes: (1) extensive temperature range, (2) wider pH range, (3) superior thermostability and (4) improved reusability. Over more than

3 years, around 25 nanoparticles have interacted with more than 40 enzymes. Some of these enzymes from microbial sources are mentioned in Table 4.2. Lysozyme is the first enzyme extensively used as a model enzyme to study the nanoparticle or nanomaterial interaction (Chen et al. 2017). The aggregation of enzymes with the nanoparticles influenced enzyme activity and loading by reducing the adsorption space and substrate availability. The enzyme laccase immobilized on carbon nanoparticles has been used for bisphenol removal. When compared to free laccase, the immobilized laccase ENP had considerably reduced reaction rates due to diffusional limitation and showed no observable structural change after enzyme immobilization (Pang et al. 2015). The immobilization of microbial cellulolytic enzymes on magnetic nanoparticles reduced the problem of steric hindrances between the carrier and enzyme and also showed remarkably high catalytic efficiency. They are more stable in storage, pH, heat and against some denaturants. They were found less inhibitory to their products and inhibitors. They have potential applications in various industries like brewing, animal feed and coffee processing, agricultural waste bioconversion, etc. Immobilized cellobiases on nanomaterials have potential as therapeutic agents (Husain 2017a). Gold nanoparticles are widely used metal nanoparticles due to their distinctive physicochemical properties and have particular interest in enzyme immobilization (Fournier et al. 2016). Nanosilica,

Table 4.2 Microbial enzymes interacted with nanoparticle

Microbial enzyme	Organism	Nanomaterial	References
Lysozyme	*Bacillus* sp.	Silver nanoparticles	Ashraf et al. (2014)
Laccase	*Rhizoctonia solani*	Carbon nanoparticles, multiwalled carbon nanotubes (MWNTs) and graphene oxide (GO)	Pang et al. (2015)
Cellulase	*Acetivibrio cellulolyticus*, *Bacillus* sp.	Magnetic nanoparticles, MWNTs	Husain (2017a)
α-Amylase	*Bacillus* sp.	Nano pore zeolite	Talebi et al. (2016)
Pullulanase	*Enterobacter aerogenes*, *Fervidobacterium pennivorans*	Magnetic nanoparticles	Husain (2017b)
Cholesterol esterase	*Candida rugosa*	Silver nanoparticles	Chawla et al. (2013)
Lipase	*Candida rugosa*	Gold nanoparticles	Venditti et al. (2015)
DNA ligase	*E. coli*	Gold nanoparticles	Wang et al. (2010)
Microbial esterase	*Enterococcus faecalis*	Cobalt oxide	Song et al. (2015)
Haloalkane dehalogenase	*Mycobacterium smegmatis*	Iron oxide nanoparticle	Koudelakova et al. (2013)

nano-ZnO, cobalt oxide nanoparticles and CdS nanoparticles are also used for immobilization of microbial enzymes. A novel pullulanase enzyme from *Fontibacillus* sp. strain DSHK107 has been immobilized on nanosilica using glutaraldehyde crosslinker and showed better thermostability (Alagöz et al. 2016). In contrast with free enzymes, nanoporous silica improved the reusability and thermostability of inulinase isolated from *Aspergillus niger* (Karimi et al. 2016). Likewise, immobilized α-amylase from *Bacillus* sp. on nano-ZnO showed enhanced thermal stability than free α-amylase (Antony et al. 2016). For immobilizing the enzyme microbial esterases, cobalt oxide nanoparticles has been widely used. The ENP retained eighty-five percentage of enzyme's original activity after 15 reuses (Jang et al. 2014).

4.3.4 Applications of ENPs

Due to high operational simplicity, low-cost fabrication, real time and rapid detection, specificity, sensitivity, selectivity, cheapness and rapid detection, currently ENPs are commercially used in the fabrication of highly sensitive, low-cost and environment-friendly products like biosensors production, biomedical devices, mini biochips, biofuel cells and enzyme reactors. ENPs-based amperometric biosensors have many valuable roles in diagnosis and treatment of diseases by measuring cholesterol, glucose, H_2O_2 and uric acid in biological fluids. Moreover, like free enzymes, ENPs are able to catalyse organic synthesis using inorganic media. It has many uses such as in pectin hydrolysis, juice production, esterification of fats oils and foods, biodiesel production, production of high-fructose corn syrup, carbon dioxide capture, etc. (Ahmad and Sardar 2014). Some applications of ENPs and their functions are listed in Table 4.3.

4.4 Future Perspectives

Enzyme nanotechnology is a fascinated and boundless area over recent years, since its innumerable applications in our day to day life and have potential impact on several scientific areas like pharmaceutical industries, food industries, energy, medicine, space industries and electronics. The interaction of enzymes with nanoparticle can modify the structure and function of enzymes. Recently, it has been widely reviewed that this enzyme modification is governed by crucial properties of nanoparticles, viz. size, structure, charge, surface chemistry and surface shape. The preparation of enzyme nanoparticles with appropriate functionalization looks an auspicious strategy to design biosensors/biomedical devices, biofuel cells and enzyme reactors. The well-designed ENPs could also be used as unique tags for protein and DNA bioaffinity assays. This novel technology is expected to expose fresh opportunities for biosensors in medical management. The miniaturized form of ENPs-based

Table 4.3 Some applications of enzyme nanoparticles

ENPs	Application	References
α-Amylase silver nanoparticles	Starch hydrolysis	Mishra et al. (2013)
Lysozyme chitosan nanoparticles	Antibacterial	Park et al. (2013)
Cellulase TiO_2 nanoparticles	Hydrolysis of carboxymethyl cellulose	Ahmad and Sardar (2014)
Glucose oxidase thiolated gold nanoparticles	Glucose level estimation	Pandey et al. (2007)
α-Chymotrypsin polystyrene nanoparticles	Proteolysis	Jia et al. (2003)
Lipase polystyrene nanoparticles	Esterification, aminolysis	Miletic et al. (2010)
Superoxide dismutase (SOD) Fe_3O_4 coated nanoparticle	Biosensors	Thandavan et al. (2013)
β-Glucosidase iron oxide nanoparticle	Biofuel production	Verma et al. (2013)
β-Galactosidase ZnO nanoparticles	Lactose hydrolysis	Ansari et al. (2011)
Laccase chitosan-magnetic nanoparticles	Bioremediation of environmental pollutants	Kalkan et al. (2012)
Alcohol dehydrogenase gold and silver nanoparticles	Alcohol synthesis	Petkova et al. (2012)
Peroxidase (BGP) TiO_2 nanoparticles	Phenol and dye removal	Ahmad et al. (2013)
Keratinase Fe_3O_4 nanoparticles	Synthesis of keratin	Konwarh et al. (2009)
Cholesterol oxidase Fe_3O_4 nanoparticles	Analysis of total cholesterol in serum	Kouassi et al. (2005)
Horseradish peroxidase (HRP) magnetite silica nanoparticles	Immunoassays	Yang et al. (2004)

sensor could provide minor, transportable, low-cost, user-friendly and extremely sensitive diagnostic devices. The improved stability could significantly multiply enzymatic catalysis at higher temperature, and this could also improve the shelf life. One of the challenging studies in the field of ENP manufacturing is sample preparation. For the complete dissolution of biological and environmental samples, ENP extraction was done by traditionally using concentrated acid solutions. In the future, simple and better extraction methods could be explored for the ease of applications in extensive fields. Enhanced understanding of transport, toxicity and exposure of ENPs primarily depends on the clear and precise characterization of ENPs. So, intensive studies are required to improve the control and productivity for the synthesis of nanoparticle. Hence, the research of ENPs in the future should explicitly be concentrated on nanometrology. It is anticipated that the practice of ENPs over free enzymes in the various industries would provide superior outcomes. The area of enzyme nanotechnology is at the state of flourishing from the last decade, and only diminutive works have been done. Consequently, there is a scope for extensive research in the field of enzyme nanoparticle technology.

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Chapter 5
Bacterial Nanowires: An Invigorating Tale for Future

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5.1 Introduction: Bacterial Nanowires – Nanostructures, More Than We Thought

Bacteria are well known to produce nanomachinery like pili, flagella, and periplasmic outgrowth. All are proteinaceous and made up of amino acid monomers (Reardon and Mueller 2013). Bacterial nanowires are an extracellular protein which is electrically conductive in nature. Electrogens are the microorganisms which can transfer electrons from cell to extracellular substances through their nanowires. Such organisms were employed in different areas like microbial fuel cell (MFC) to produce green energy (Das and Mangwani 2010), other fuel production (Du et al. 2007), and bioremediation (Du et al. 2007).

For the past decades, the world has been looking for different fields of energy generation. All our major energy generation sectors are almost entirely dependent on nonrenewable energy source, which is a major concern of the present scenario. MFC plays an important role in renewable energy production as it converts the chemical energy into electrical energy (He et al. 2005; Siegert et al. 2019; Shah et al. 2019). Various substrates can be used in MFCs to produce green energy (Pant et al. 2010) (Fig. 5.1). Bacterial nanowires serve as a bridging material between the electrogens and the anode of MFC (Ntarlagiannis et al. 2007). Bacterial nanowires also help the biofilm formation on anode which enhances the electron transport in MFC (Malvankar and Lovley 2014). In addition to the application in MFC, bacterial nanowires are also employed in bioelectronics and biosensor production, among other areas. In this chapter, we take an effort to discuss all the scientific potential of bacterial nanowire research with special emphasis on its structural diversity, molecular manipulation, bioenergy production, bioremediation, and bioelectronics.

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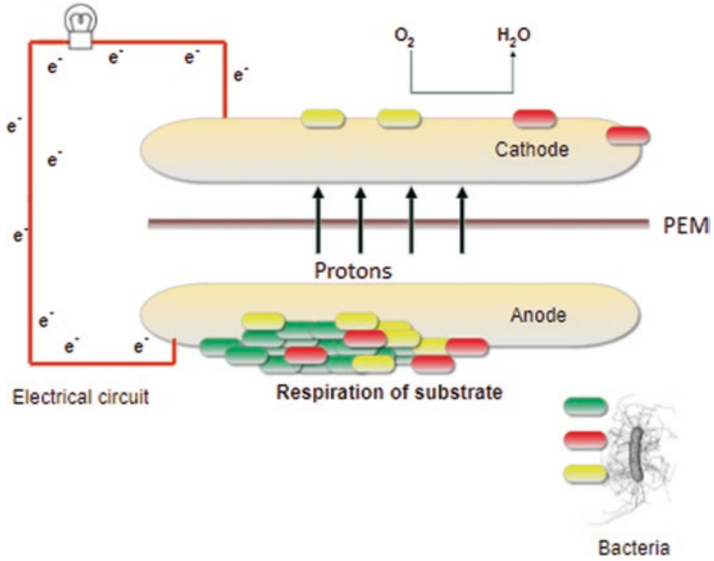

Fig. 5.1 Schematic representation of MFC: Green energy production using MFC

5.2 Milestones in Bacterial Nanowire Research

Some bacteria can transfer electrons from cell to outer environmental extracellular electron carrier during anaerobic respiration. Such energy-extracting process is known as extracellular respiration (Sure et al. 2016a). Metal-reducing bacteria like *Geobactor sulfurreducens* and *Shewanella oneidensis* are good examples of microorganisms which can perform extracellular electron transfer. Exoelectrogen is the common term used to describe such microorganisms that have the ability to transport electrons extracellularly, utilizing three common strategies: (i) exoelectrogens transfer electrons directly to electron acceptor through outer membrane proteins; (ii) some mediators like metal chelators, dyes, and pigments act like a puppet master to transfer electrons from cell to outer electron carrier; and (iii) the bacterial nanowires can act as a circuit through which the energy is transferred from the living system to outer electron acceptor (Fig. 5.2).

The conductive nature and direct role in extracellular electron transfer of conductive proteins were not revealed in early times of research. Even extracellular pili-like structures (PLSs) of various exoelectrogens like *Shewanella* and *Geobacter* were also believed as nonconductive (Sure et al. 2016b). But later it was experimentally evidenced that PLSs of some *Shewanella* spp. are electrically conductive (Pirbadian et al. 2014). Detection and identification of the presence and role of bacterial nanowires seemed to be of little difficulty in early times due to several possible reasons. The culture used for cultivation is an important factor. Complex

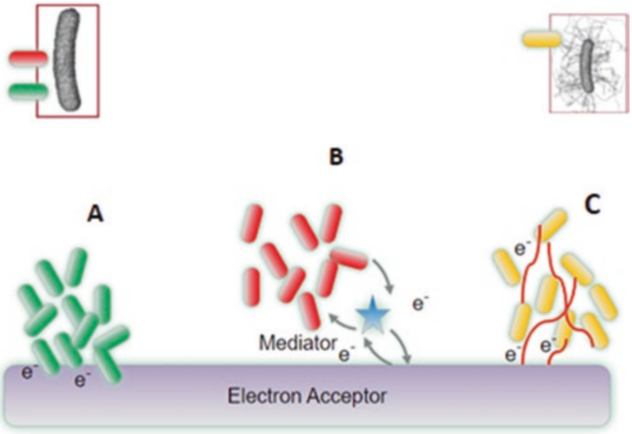

Fig. 5.2 Electron transfer strategies of electrogens: Electrogens can transfer electrons by direct attachment to electron acceptor (**a**) or through mediators (**b**) or can employ bacterial nanowires (**c**)

formulations, such as Luria-Bertani, were used in early studies (Reguera et al. 2005), which limited the extracellular electron transfer. The particular nature of pili and diverse types of PLSs also led to delay the proper identification of the role of bacterial nanowires in electron transfer. Development of research led to the discovery of various exoelectrogens which can oxidize and reduce vivid extracellular substances present in its external environment. With this identification, a novel hypothesis about bacterial nanowires describing its capability to connect cells with extracellular electron donor and acceptor was proposed. Not only exoelectrogen candidates which are mentioned here but also many other candidates were identified as a result of further efforts and research. The presence of bacterial nanowires in exoelectrogens was also confirmed using various techniques in nanotechnology such as different modes of atomic force microscopy, scanning tunneling microscopy, and electrostatic force microscopy (Sure et al. 2016b). The major exoelectrogens which can produce bacterial nanowires are shown in Table 5.1 (Sure et al. 2016b).

5.3 Bacterial Nanowire Diversity

Various types of exoelectrogens which can produce nanowire were identified so far (Table 5.1). Each and every species differ in its structure and composition of nanowires. Based on the available information, bacterial nanowires can be classified as follows.

Table 5.1 The major exoelectrogens which produce bacterial nanowires

Exoelectrogens	Protein component	Physiological Role	Conductivity	References
I. Metal reducing				
G. sulfurreducens	Pilin subunit Pili A	Extracellular electron transfer	Along the length and width	
S. oneidensis MR1	Periplasmic extension with cytochromes	Unknown	Along the length and width	Pirbadian et al. (2014)
D. desulfuricans	Unknown	Unknown	Along the width	Eaktasang et al. (2016)
II. Photosynthetic				
Synechocystis sp. PCC 6803	Pilin subunit Pili A	Unknown	Along the width	Sure et al. (2015)
Mi.aeruginosa	Unnamed protein (GenBank:CAO90693.1)	Unknown	Along the width	Sure et al. (2015)
R. palustris RP2	Unknown	Unknown	Along the length and width	Venkidusamy et al. (2015)
No. punctiforme	Unknown	Unknown	Along the width	Sure et al. (2016b)
III. Chemoautotrophs				
Aci. ferrooxidans	Unknown	Unknown	Along the width	Carmona et al. (2014)

5.3.1 Pili

A pilus is a surface structure found in many bacteria. Common types of pili are conjugate pili, which usually participated in gene exchange between two bacteria and Type IV pili (T4P), which is responsible for the generation of motive force and electrical conductance (Feliciano et al. 2015). T4P is the most widespread type of pili in exoelectrogens. We can undoubtedly consider T4P as a multifunctional extracellular structure because its role in the physiological process is diverse such as cell adhesion, motility, DNA exchange, biofilm formation, and most deliberately the electron transport.

The structural and dimensional properties of pili are varying with different exoelectrogens. Table 5.2 shows a comparison of *Geobacter sulfurreducense* and *Synechocystis* (Malvankar and Lovley 2014; Reardon and Mueller 2013). T4P has bundle formation capacity which alters the width of pili. Sample preparation method and the age of culture can affect the length of pili also. Hence, the length and width of the pili are different under different cultural conditions.

5.3.2 Other Extracellular Proteins

Msh pili and flagella are other extracellular structures in addition to T4P pili in exoelectrogens. Various deletion mutation analysis confirms the role of such extracellular structures in extracellular electron transfer. But its exact role as bacterial nanowires is still unknown (Sure et al. 2016b).

Table 5.2 Structure and function of Pili in *Geobacter sulfurreducense* and Synechocystis

Exo electrogens	Subunit of Bacterial nanowire	Associated protein	Molecular mass of subunit	Dimensions	References
G. sulfurreducense	PiliA	Cytochromes	~10 kDa	Width/length: 3–5 nm/ 10–20 μm	Lovley (2011)
Synechocystis	PiliA1	Unknown	~20 kDa	Width/length: 4.5–7 nm/2–10 μm	Lovley (2011)

Table 5.3 Exoelectrogens producing unknown pili-like nanowires

Exoelectrogens	Descriptions	References
No. punctiforme	Two distinct types of nanowires (a) Short/thin BNWs of size 6–7.5 nm in diameter and 0.5–2 μm in length (b) Long/thick BNWs of size ~20–40 nm in diameter and ~10 μm long	Sure et al. (2016a)
Mi. aeruginosa	Composed of a protein similar to an unnamed protein (GenBank: CAO90693.1)	Sure et al. (2016a)
Pe. thermopropionicum	Produces electrically conductive flagellum-like appendages (10–20 nm in diameter)	Gorby et al. (2006)
G. sulfurreducens	Produce flagella which were found to be nonconductive	

5.3.3 *Mysterious Conductive Structures*

Table 5.3 describes the details of the other unknown pili-like conductive structures, which have been identified in different microbial species. Further functional and structural analysis is required to reveal their mysterious role in electron transport to the outer environment.

5.4 A Bacterial Nanowire, as a Vital Organ

As each and every bacterial nanowire was originated as a physiological requirement of specific bacteria, it is not necessary that they should share a common structural strategy. And hence each specific bacterial nanowire has its own specific structural composition. As mentioned earlier, the complete structural and functional elucidation of bacterial nanowires is still under development. The most commonly discussed functions are as follows.

5.4.1 As a Corridor in Electron Transfer

The most widely accepted hypothesis about the functions of the bacterial nanowire is that it acts as a bridge in electron transfer between a cell and an external substance as well as between two living cells.

5.4.1.1 Between Cell and Outer Electron Acceptor/Donor

Bacterial nanowire acts as a mediator for the bidirectional transfer of electrons. For example, in metal-reducing bacteria, bacterial nanowire helps to transfer the electrons to an extracellular electron acceptor (Sure et al. 2016a), and in a metal-oxidizing organism, it helps to transfer the electrons toward the cell itself (Reguera et al. 2005).

5.4.1.2 Between Two Specific Cells

Without any external intermediate for electron transfer, the microorganism can transfer electrons from one living cell to another as interspecific (between two species of the same genus) electron transfer as well as an intergeneric (between two genera) electron transfer. The various interspecific and intergeneric electron transfers are shown in Table 5.4.

Table 5.4 Electron transfer with bacterial nanowires

Exo electrogens	Descriptions	References
I. Between cell and electron acceptor		
G. sulfurreducens	Direct transfer to metal	Reguera et al. (2005)
Aci. Ferrooxidans	Direct transfer to metal	Carmona et al. (2014)
Synechocystis	Direct transfer to metal	Sure et al. (2016a)
II. Interspecies electron transfer		
G. metallireducens *G. sulfurreducens*	Directly transfer electrons to each other, rather than use hydrogen and formate as intermediate electron carriers	Morita et al. (2011)
III. Inter-generic electron transfer		
Eubacteria and *Archaea*	A part of symbiotic microbial consortium	Wegener et al. (2015)
Cyanobacteria and other microbes	Cyanobacteria transfer electrons to other microbes in the microbial mat	Lea-Smith et al. (2016)

5.4.2 *As an SOS Factor in* Cyanobacteria

Bacterial nanowires stimulate plastoquinone to donate electron during photophosphorylation in cyanobacteria. This stimulation and the extra electron donation of plastoquinone reduce the risk of cell damage due to the poor electron flow during the carbon-limiting condition.

5.5 Bacterial Nanowires as an Electron Shuttling Gadget

Various mechanisms were proposed in order to describe the actual mechanism of electron transfer through conductive proteins. Experimental analysis finally suggests two major mechanisms can be possible for transferring the charged particles through a conductive protein and they are the metallic-like conductive model and the electron-hopping model (Morita and Kimura 2003).

5.5.1 The Metallic-Like Conductive Model of Electron Transport

The experimental analysis in *G. sulfurreducens* revealed that the bacterial nanowires have metallic-like electrical conductivity as in some organic conductors (Bai et al. 2016). But most of the biological system shows electron-hopping mechanism for electron transfer; hence, this metallic-like electrical conductivity model seems to be distinct (Sure et al. 2016a). The following identifications will support the metallic-like conductivity model of electron transfer in bacterial nanowires.

5.5.1.1 Acid-Base-Dependent Electrical Conductivity

pH changes induce the conformational changes in individual amino acids, which may alter the conductive nature of proteins. Experimental studies in *Geobacter* supported the above-mentioned fact as the conductivity of pili increases during low pH due to the conformational change in individual aromatic amino acids (Malvankar et al. 2015).

5.5.1.2 Role of Aromatic Amino Acids in Electrical Conductivity

The overlapping π-π orbitals of the aromatic ring are one of the most contributing factors for electron transfer in synthetic materials. Pilin-like protein also contains aromatic amino acids with overlapping π-π orbitals, and hence in both the case of synthetic organic materials and conductive proteins, electron transfer mechanism seems to be same. Various deletion and substitution mutation analyses were performed to confirm the role of aromatic amino acids in electron transfer. For example, the *Geobacter Aro-5*, a genetically modified bacteria, with five aromatic amino acids in pili A, one of the subunits of pili was replaced with alanine (Sure et al. 2016b). In this study, the electron conductivity was found to be reduced in transformed bacteria when compared to the wild type. It is also clear that the conformational change alters the functional properties of proteins. Hence the removal of aromatic amino acids definitely alters the three-dimensional structure, which may lead to the change in conductivity of pili (Vargas et al. 2013).

In addition to this, molecular tilting and the interplanar distances also have a role in the electron transport. The studies of pili on *Neisseria gonorrhoeae* show that the aromatic amino acids are placed too far (Yan et al. 2015). But the further experiments using techniques like synchrotron X-ray microdiffraction and rocking-curve X-ray diffraction in *Geobacter* pili again proved the role of aromatic amino acids in electron transfer even in long-range distance (Malvankar et al. 2015).

5.5.1.3 In Silico Modeling Analysis

Modeling studies also proved that the metallic-like conductivity model suggests the lowest energy model in *Geobacter*. It suggests that a close-packed central core chain of aromatic monomers facilitated the electron transport along the entire length of the pilus (Xiao et al. 2016). The gene deletion study on *G. sulfurreducens* also reveals the role of the pilus structure in electron transport (Liu et al. 2014). From all the above-observed findings, scientists argue that the electron transfer mechanism in bacterial nanowires is the metallic-like conductivity model (Sure et al. 2016b)

5.5.2 *Electron-Hopping Model*

Another emerging view on electron transport in exoelectrogens like *Geobacter* and *Shewanella* is the electron-hopping model. Aromatic amino acids are the central attraction factor in electron transport of bacterial nanowires of *Geobacter* (Yan et al. 2015), but in *Shewanella*, it is believed that cytochromes have the central role in electron transport (Gorby et al. 2006). Whatever may be the crucial factor in electron transport, the mechanism followed to transport the charged particle through the conductive bacterial nanowires of any organism is still debatable. It can be by the electron-hopping model or by the previously described metallic-like conductive model.

Various in silico modeling studies strongly supported the electron-hopping model of electron transfer in bacterial nanowires of *Geobacter* (Feliciano et al. 2012; Yan et al. 2015). Another experimental evidence has come in order to substantiate the electron-hopping model of electron transport in *Geobacter*, such that at low voltage cryogenic STM of *Geobacter* pili showed the thermal activation of the differential transversal conductance, which is in accordance with electron-hopping mechanism (Sure et al. 2016a).

5.6 The Potential Suits of Microbial Nanowires

The benefits of an object make it more popular. The applications of bacterial nanowires are widespread in vivid areas. Here we are discussing the major applications of bacterial nanowires which are supposed to be the major boom of the coming era.

5.6.1 Green Energy Production

Microbial fuel cell is a promising technology to generate green energy. In microbial fuel cell, chemical energy is converted to electrical energy, through the action of microorganisms called electrogens. Technically an MFC consists of an anode and a cathode, and sometimes, it needs a membrane (Fig. 5.2). It utilizes a different substrate as fuel to produce green electricity with oxidation-reduction reaction.

Biofilm formation is one of the most critical factors in MFC which enhances the electron flow even between distantly located substrates or electrogens, and thereby it increases the efficiency of power generation (Du et al. 2007). Bacterial nanowires help electrogens to make biofilm (Steidl et al. 2016), and thereby, it increases the power generation efficiency of MFC (Das and Mangwani 2010). Bacterial nanowires also act as a bridge between electrogens and electrode as in *Geobacter sulfurreducens* (Sure et al. 2016b), which facilitate the long-range electron transfer and there by energy production (Steidl et al. 2016).

5.6.2 Fuel Generation

Bacterial nanowires can be employed for the production of hydrogen and methane-like fuels, in MFC with a slight modification. Generally, in a MFC, the proton and electrons were produced in anode, which are then transported to cathode, and from cathode they were combined with oxygen to form water. Thermodynamically the hydrogen production in such ways is not feasible. But in a MFC, the increased cathode potential overcomes the energy barrier which may lead to hydrogen gas production by combining the proton and the electron. The experimental analysis suggests that MFC can probably produce hydrogen as the amount of hydrogen produced in glucose fermentation method (Zhou et al. 2013).

Anaerobic digestion of some wastewater and biomass produces methane as a secondary product in MFC (Wegener et al. 2015). MFC with little modification produces methane in the cathode chamber. Here also the increased cathode potential facilitates to cross the thermodynamic barrier to produce methane in the cathode (Zhou et al. 2013). Bacterial nanowires of exoelectrogens play a crucial role in both hydrogen production and methane production in MFC as an electron shuttle mediator between the bacteria and the anode (Sure et al. 2016a).

5.6.3 Bioremediation

Uses of electrogens and their bacterial nanowires to treat the contaminated water and the biomass were successfully employed in MFC. As the waste waters are rich sources of carbon and other organic substances, it can definitely act as an ideal substrate in MFC. In addition to organic waste, bacterial nanowires are particularly employed to treat the heavy metal contamination like uranium contamination (Cologgi et al. 2011). Gene deletion mutation analysis on *Geobacter* proved that the presence of bacterial nanowires in electrogens increases the efficiency of uranium contamination treatment (Cologgi et al. 2011). Bacterial nanowires help in different ways to treat the heavy metal contaminations, such as it increases the surface area and thereby increases the bioavailability of uranium for absorption (Cologgi et al. 2011) and it increases the cellular tolerance to heavy metal by preventing the cellular accumulation (Cologgi et al. 2011). In addition to uranium, bacterial nanowires also help to precipitate arsenic, chromium like metal and there to facilitate the bioremediation process of heavy metals (Sure et al. 2016b).

5.6.4 Green Electronics

The maximum conductive nature of the bacterial nanowires makes them a delightful substance for the construction of electron transport materials, electronic devices, and sensors for medical or environmental applications. Experimental characterization of bacterial nanowires in *Shewanella oneidensis* shows that it is capable of being used as building blocks for constructing electronic devices (Lovley and Malvankar 2015). Protein engineering or molecular manipulation of bacterial nanowires may alter the conductive nature which ultimately leads to the change in the electric behavior of the organism (Tan et al. 2016). Protein engineering studies have been performed in *Geobacter*, in order to increase its conductivity especially for the usage in the bioelectronics field, such as C-terminal modification of pili A protein (Lovley and Malvankar 2015), which reduces the diameter of pili and their by increases the conductivity.

5.7 Future Directions

From all the findings, it is clear that microbes may develop bacterial nanowires according to their physiological needs. Extensive and elaborate research is required to study the needs and mechanism of development of bacterial nanowires, which may reveal the exact role of nanowires in nature. In order to identify the super candidate among the known electrogens, molecular, biochemical, and mechanical characterization of all bacterial nanowires which are known so far should be done. Extensive researches are also needed in the field of electron transport mechanism, simple ways for the culturing of electrogens and the over production of bacterial nanowires. The most important fact that a few laboratories are involved in bacterial nanowire research till to date. The identical or similar research data on bacterial nanowires from other laboratories definitely increases the authenticity and the scope of bacterial nanowire research. Understanding the knack of nanowire electric work has applications well beyond the discussion. Such structures have the potential to address some of the big questions about the nature of life itself.

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Chapter 6
Antimicrobial Silver Nanoparticles: Future of Nanomaterials

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6.1 Introduction

Microorganisms are everywhere, around us and deeply within us. Some of them are beneficial and while other cause major sepsis, mortality and further, modernization is enhancing these challenging issues. Despite immense advancement in diagnosis and treatment, the microbial infections are acknowledged cause of chronic sepsis, morbidity, and mortality. Available reports showed that more than 3 billon people die due to microbial infection and their contamination in developing countries (Baranwal et al. 2018). The emergence of bacterial resistance toward antibiotics is becoming the major challenging conditions. This issue has led to the emergence of multidrug resistance (MDR) bacterial strain and superbacteria, which shows resistance to nearly all antibiotics. The reason behind this is the wide use and abuse of antibiotic which leads to evolutionary process in bacteria, both genetically and morphologically (Wang et al. 2017). The mechanism of resistance includes expression of efflux pumps, modification of cell components, and expression of some enzymes such as aminoglycosides. Further, a gene NDM-1 (New Delhi metallo-β-lactamase) is a supergene that is responsible for broad-spectrum antibiotic resistance in superbacteria (Hsueh 2010). Apart from these, most bacterial species interact with each other to form microbial aggregates on a solid surface and extracellular polymeric substance (EPS) which is secreted outside by these bacteria itself. These bacterial aggregates on EPS are specifically known as biofilm. These biofilms act as a protective covering for the bacteria in unfavorable environmental conditions and act as a barrier for antibiotics which inhibit their access to the bacteria that leads to continuous growth resulting in a mature biofilm. Further, these mature biofilm bacteria can invade the immune system by producing the superantigen within the biofilm.

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Thus, these biofilms are also a serious threat to biomedical and healthcare section (Arima et al. 2018; Zhu et al. 2018).

To combat these issues of microbial resistance and biofilm, recent advancement in nanostructured formulation has occurred that is raising a hope in antimicrobial medication. The most accepted mechanism of nanostructure formulation, nanoparticles (NPs), is that they directly contact with the bacterial cell wall and destabilize them. These NPs do not need to penetrate inside the cell wall and this is the reason that the antibiotic resistance mechanisms are irrelevant for these particles (Edmundson et al. 2013). These NPs, both organic and inorganic, have antibacterial properties against a wide range of bacteria. Among all the silver nanoparticles (AgNPs), the inorganic one is of great interest of researchers as antimicrobial agent because of their effectiveness, versatile applications, and potential to enhance the effect of conventional antibacterial agents toward multidrug resistance bacteria by synergism (Siddiqi et al. 2018; Aziz et al. 2014, 2015, 2016). If we traced back in history, before the introduction of antibiotics, silver in various forms (metallic silver, silver salt and silver colloids) has been extensively used throughout for a variety of configuration including vessels, water preserver in the form of liquid container and silver coins, shaving, sutures, and medicinal purposes for which it was first documented in 750 AD (Alexander 2009; Maillard and Hartemann 2013).

The journey of silver to silver nanoparticles occurs due to dynamic development of nanotechnology. Earlier silver has been used in the form of silver nitrate, silver sulfadiazine, and silver zeolite (Rai et al. 2012). The advantage of AgNPs over silver salt is due to their unique property of having highly developed and larger surface area to volume ratio which leads to higher bioavailability and allows increased antimicrobial activity than the bulk material. Further, silver nanoparticles have been proven to show less toxicity in comparison to silver (in its ionic form), even at same molar concentration. The antibacterial potential of AgNPs has high influence based on their physicochemical properties, i.e., size, morphology, charge, and stability. These physicochemical properties further depend on the method of their synthesis and preparation condition like nature of solvent, strength of reducing agent, concentration, and temperature (Kedziora et al. 2018).

According to the market survey, AgNPs, among all nanomaterials, is the leading commercialized nanoparticles that accounts for more than 50% of the global nanomaterial consumer products. It is estimated that the annual manufacture market of AgNPs goes up to 320 tons production which have been used for healthcare and food products only and the global market is known to surpass over 3 billion by 2020. Due to its unique antibacterial and antifungal properties, their preference is rising in textile, water treatment, agriculture, paint industry, and food and beverage packing industry (Adibzadeh et al. 2014; Ahmadi and Adibhesami 2017; Li et al. 2017). Various approaches are available for their production that is broadly categorized into two groups, i.e., top-down and bottom-up (Fig. 6.1).

Top-down method includes physical processes that include the breakdown of solid into nanoparticles, whereas bottom-up method includes synthesis of nanoparticles from silver ions by employing chemical, photochemical, and biological reactions.

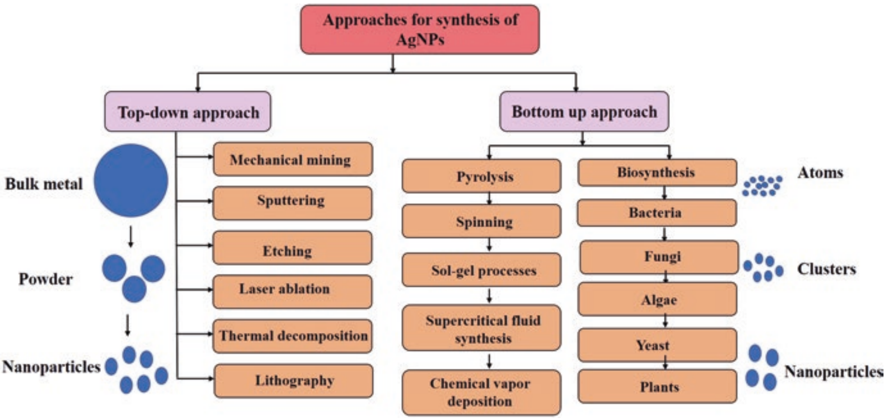

Fig. 6.1 Various approaches that have been used to produce silver nanoparticles

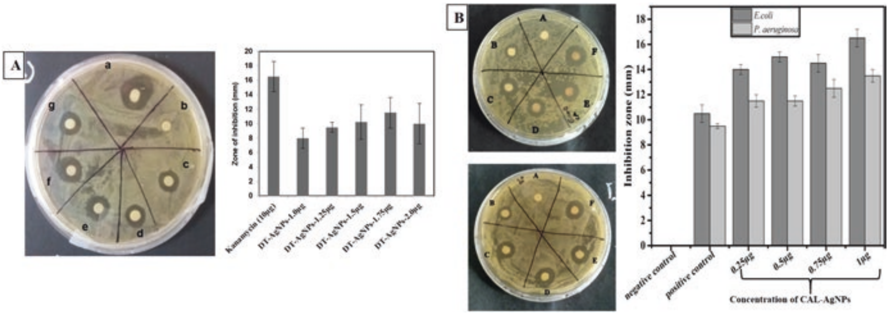

Fig. 6.2 Antibacterial potential of Biosynthesized AgNPs from (**a**) Bark extract of *Dicoma tomentosa* against *P. aeruginosa*. (**b**) Leaf extract of *Cicer arientinum* against *E. coli* and *P. aeruginosa*

The physical and chemical methods of synthesis are expensive, generate toxic by-products, and have high-energy requirements. To mask these issues of toxicity, biological methods that include microorganism and plant-based synthesis have evolved as one of the most promising options in the AgNP biosynthesis (Singh et al. 2016; Prasad 2014). Among them, plant-based synthesis of AgNPs is considered as very cost-effective and less laborious (Joshi et al. 2018). Several Phyto-constituents possesses both protective and reductive properties that are mainly responsible for the reduction of silver ions to AgNPs. Several studies have been done for biosynthesis of AgNPs from plant extracts including *Rheum turkestanicum*, *Salvia lerifolia*, *Terminalia arjuna*, *Prosopis juliflora*, *Canarium ovatum*, *Cicer arientinum*, *Cordia dicotoma*, and *Dicoma tomentosa*. Here in these studies, the synthesized AgNPs showed very efficient antimicrobial potential (Fig. 6.2) (Ahmed et al. 2017; Arya et al. 2016, 2017a, b; Baghayeri et al. 2018; Geeta et al. 2017; Kumari et al. 2016; Taghavizadeh Yazdi et al. 2018).

In the present chapter, we will focus and discuss the current scenario and recent advancement of silver nanoparticles as antimicrobial agent in widespread domains such as healthcare, textile, and water treatment. Further, we will discuss their possible challenges and future prospects.

6.2 Morphological Classification of Silver Nanoparticles Along with Their Antimicrobial Effect

By reaching to a nanometer platform, silver tends to show unique properties and a strong correlation exists between morphology (shape and size) and physical properties of silver nanoparticles on biological platform. Cellular uptake, cellular activation, intracellular distribution and the antibacterial potential of these nanoparticles are highly influenced depending on the shape, size, and surface charge of these nanoparticles. Further, the size, morphology, and stability of AgNPs are strongly influenced by various parameters of reaction including methods of synthesis, reducing agent, and stabilizing agent. So, by controlling specific parameters, we can control the specific physicochemical properties of our particles. The nanoparticles synthesis reaction consists of three steps, (i) nucleation, (ii) seeding or coalescence, and (iii) growth (Fig. 6.3) in the solution, and by changing the kinetic and

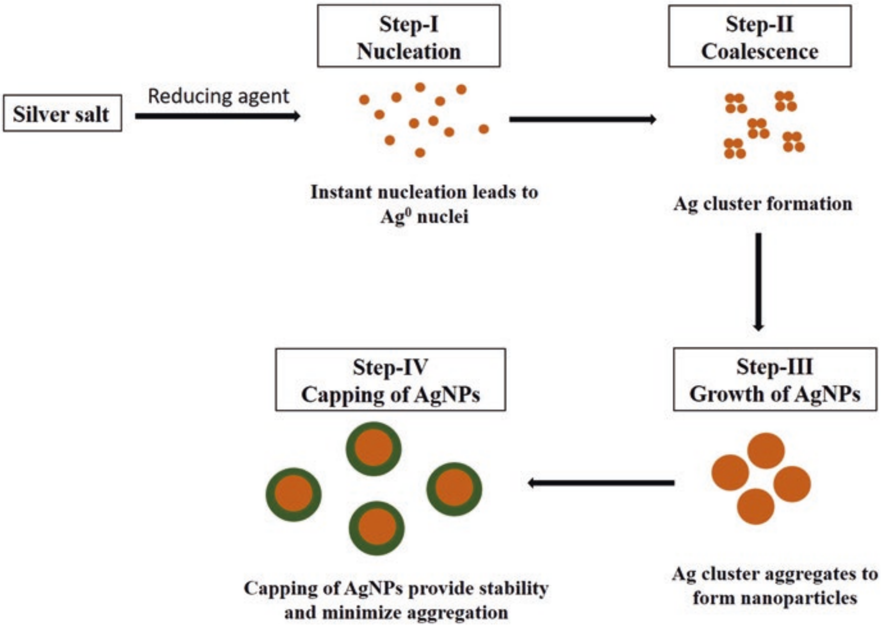

Fig. 6.3 Schematic representation of various step of development of silver nanoparticles

thermodynamics of reaction at each stage, we can control the size and shape of the particles.

Here is a brief description of factors that affects the antimicrobial potential of these silver nanoparticles.

6.2.1 Size of the Particles

The size of nanoparticles is the unique characterization which is one of the key deciding factors for their antimicrobial activity. The smaller particles have larger surface area-to-volume ratio for the interaction of microorganism and show better antimicrobial activity. A reduction of size range of particles from 10 μm to 10 nm leads to increase the interaction by increasing the surface area to 10^9 (Pal et al. 2007). Ample amount of studies has been conducted to evaluate the size-dependent antimicrobial potential of synthesized AgNPs.

Agnihotri et al. performed the antimicrobial efficacy experiment with size-controlled silver nanoparticles over the range of 5–100 nm. Here the minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC) were performed against eight bacteria including four strains of *E. coli*, two strains of *B. subtilis*, and two strains of *S. aureus*. The results of their experiment revealed that among all sizes, the best results for antibacterial effect were obtained from 5, 7, and 10 nm, and among them 5 nm demonstrated fastest results against all the test strains (Agnihotri et al. 2014). Raza et al. synthesized particles in the set of S1, S2, S3, S4, and S5 which were in the size range of 30–80, 150, 25–70, 15–50, and 30–200 nm. They performed disk diffusion assay against *E. coli* and *Pseudomonas aeruginosa*. They observed that all synthesized particles showed good antibacterial effect, but the maximum zone of inhibition (ZOI), i.e., 8 mm, was obtained through S4 sample after antibiotic ZOI (11.3 mm) due to their smaller size range (Raza et al. 2016). After that, Kim et al. showed the size effective studies against growth of bacteria. They synthesized different size ranges of particles with 70, 20, and 10 nm of spherical shape and evaluated against GFP-expressing *E. coli* for their comparative antimicrobial effect. The maximum zone of inhibition was observed with 10 nm treatment. Further, they performed comparative study for shape-dependent antimicrobial effect (Kim et al. 2017b). Further, Dogru et al. used green method where different size of particles was synthesized using extract of *Matricaria chamomilla* reduction. The particles were synthesized with 70 ± 5, 52 ± 5, and 37 ± 5 nm and tested against *S. aureus* and *E. coli*. Here the results demonstrated that particles with all three ranges showed inhibitory effect even at 6.25 ppm and among these, particle with 37 ± 5 showed highest inhibitory potential (Dogru et al. 2017).

All the studies mentioned here are satisfactory to the previous studies where finer AgNPs tend to have better antimicrobial activity than the larger one. But some exceptions are still found in the study of Ginjupalli et al. where they synthesized particles in different set of ranges including 10–20 nm, 30–50 nm, 50–80 nm, and 80–100 nm; they performed disk-diffusion assay against *E. coli* and *Staphylococcus* *aureus*, and the obtained results here revealed that the particles having 80–100 nm size only showed antibacterial potential in dose-dependent manner and other size of particles does not show the activity (Ginjupalli et al. 2018).

6.2.2 Shape of the Particles

The antibacterial potential of AgNPs not only be influenced by size; it is highly influenced by their shape too. The particles can be synthesized in different shapes including spherical, near spherical, nano-cubes, nanorods, triangular or pyramid, truncated triangular, nanowires, nano-bars, nano-prism, flower shaped, octahedral and tetrahedral (Chang et al. 2011; Xia et al. 2013).

Gao et al. synthesized silver nanoparticles with triangle, nanoplate, and nanosphere through chemical reduction method where L-ascorbic acid is used as reductant and polyvinyl pyrrolidone (PVP) as surface modification or protectant agent. These three different shaped AgNPs were then compared for their antibacterial potential against *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* through both disk diffusion and broth dilution method. Here the results indicate that the nanosphere exhibits better antibacterial potential as compared to Ag triangles and Ag nanoplates (Gao et al. 2013). In the study of Logaranjan et al., they synthesized octahedral AgNPs by biological method where *aloe vera* plant extract was used as reducing agent at room temperature and under microwave irradiation. The resulted particles were synthesized in the size range of 5–50 nm and compared to chemically synthesized nanoparticles for their antimicrobial effect against *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus luteus*, *Klebsiella pneumoniae*, and *E. coli.* The obtained results here revealed that the octahedral particles are showing two- to fourfold higher activity as compared to other particles (Logaranjan et al. 2016).

Helmlinger et al. also experimentally showed that different morphology of silver nanoparticles shows different extents of antimicrobial effect. Here the results showed that nanoplates showed higher toxicity toward bacteria in comparison to nanosphere and nanorods. Nanocubes among all these served as somewhat less effective against bacteria (Helmlinger et al. 2016).

In 2017, the study of Kim et al. as mentioned above after size effect they performed comparative studies of antibacterial effect with respect to their shape or morphology against GFP-expressing *E. coli*. Here synthesized particles were in cube, triangular plate, and spherical shape, and the size range of all the shapes was in 70 nm. The results of disk diffusion assay evaluate that the triangular plate AgNPs showed the highest ZOI comparative to the cubic and spherical nanoparticles. Further, the MIC and MBC results showed that 150 ng/ml dose of spherical- and plate-shaped nanoparticles inhibits the bacterial growth completely, whereas the required dose of cubic AgNPs was 1500 ng/ml for complete inhibition. So, their study revealed the pattern of antimicrobial effect with respect to shape is triangular plate NPs > spherical > cube (Kim et al. 2017b).

Further, Chakraborty et al. did experimental studies in which they suggested that the shape of the synthesized nanoparticles can be tuned by using protein in synthesis and protection or capping process. They used eight different proteins including lysozyme, apo-transferrin, avidin, β-lactoglobulin, BSA, hemoglobin, ovalbumin, and catalase. For the synthesis of different shaped particles, firstly Ag^+ were reduced to Ag cluster through citric acid salt. The clusters were then mixed with different proteins for AgNP growth. Further in the same pattern, particles were synthesized in the presence of different amino acids for investigation of the role of individual amino acid in shape control mechanism. The results here showed that alanine, glycine, isoleucine, serine, threonine, and valine dictate anisotropic nanoparticles, whereas lysine and cystine lead to broad size distributed spherical particles. The rest of amino acid only showed the nonsignificant optical featured reaction product. Further, among all amino acid protected AgNPs, only lysine protection (other degrades within 2 hour of synthesis) is the one, which resulted in the formation of chemically stable particles. Further, it was observed that free lysine and lysine in the protein affect differently on the shape and can lead to anisotropic growth. To evaluate this effect, a number of experiments were performed that revealed a high number of accessible lysines are responsible for anisotropic growth of nanoparticles (Chakraborty et al. 2018).

Recently, Acharya et al. in 2018 have synthesized particles using chemical reduction method with spherical and rod shapes. They performed disk diffusion method and MIC to compare their antimicrobial effect against *E. coli*, *K. pneumoniae*, *B. subtilis*, *P. aeruginosa*, and *S. aureus*. They found that both the candidates showed good antibacterial activity and in case of *K. pneumoniae* the spherical-shaped AgNPs showed higher activity as compared to rod-shaped AgNPs. The results also revealed that in case of both spherical and rod AgNPs, the (111) plane is responsible for their antimicrobial effect (Acharya et al. 2018).

6.2.3 Electric Charge on the Surface of Silver Nanoparticles

The electric charge on the surface of AgNPs tends to contribute effective variation on the potential bioactivity of the particles. Depending on the method of synthesis, different surface charges can exhibit on the surface of particles. Generally, the stabilizer and the capping agent in the synthesis procedure are employed to change the surface charge that can provide stability and influence the bioactivity of the AgNPs.

Silva et al. synthesized organo-coated silver nanoparticles having positive, negative, and highly negative charge on the surface. These three coated AgNPs were further compared with $AgNO_3$. Here, for synthesis of coated charged AgNPs, polyvinyl pyrrolidone (PVP), citrate, and branched polyethyleneimine (BPEI) were employed as stabilizers that were responsible for negative (−7), highly negative (−20), and positive charge (+28) AgNPs, respectively. Further, these particles were evaluated for comparative study of their antibacterial potential against two model bacteria, i.e., *E. coli and Daphnia magna*. The results here indicate that among all the three charged coated nanoparticles, BPEI-AgNPs showed the maximum antibacterial potential by *E. coli* bioassay (% inhibition of β-galactosidase) and *Daphnia magna* mortality assay (% of mortality). The reason behind this was the smaller particle size of the particle and the most importantly the positive charge on the surface, i.e., +28 that leads to effective electrostatic interaction of particles to the cell membrane of bacteria. In case of *E. coli*, the EC_{50} (effective concentration) value of BPEI-AgNPs, citrate-AgNPs, and PVP-AgNPs were evaluated as 305, 791, and 2040 µg/L, respectively. Further, in case of *Daphnia magna*, the LC_{50} values (median lethal concentration) were observed as 0.45, 2.69, and 4.71 µg/L with respect to above (Silva et al. 2014).

Further, the study was also supported by Abbaszadegan et al. They conducted the study to evaluate the effect of neutral AgNPs for their antimicrobial potential in comparison to negatively and positively charged AgNPs. Here they investigate their study against Gram-positive (*Staphylococcus aureus* and *Streptococcus mutans*) and Gram-negative (*E. coli* and *Proteus vulgaris*) bacteria. For this study, they firstly synthesized AgNPs with different surface charges. For negatively charged particles, sodium borohydride was used as a reductant and rice starch was used for the synthesis of neutral AgNPs. For positively charged particle synthesis, AgNO3 were mixed with 1-dodecyl-3-methylimidazolium chloride and sodium borohydride was added. Further to investigate antibacterial potential, both well diffusion and microdilution method were performed against the panel of bacteria mentioned above. From the results, it was evaluated that in well diffusion assay, the highest inhibition zone was presented by the positively charged AgNPs against all test organism, in all concentrations. Further microdilution (MIC) results suggested that the positively charged AgNPs are much active and effective at 100 to 1000 times lower concentrations as compared to negative and neutral AgNPs and even the size of these particles (i.e., 19 nm and 20 nm, respectively) are smaller than positively charged particles (24 nm) (Abbaszadegan et al. 2015).

In contrast to this, Salvioni et al. (2017) conducted a study where they synthesized water-soluble, negatively charged AgNPs through the reduction of citric acid and tannic acid. They assessed their synthesized AgNPs against Gram-negative *E. coli* MG1665 and Gram-positive *Staphylococcus* bacteria for MIC and compared to the commercially available AgNPs, i.e., citrate-stabilized AgNPs, CSNPs (colloidal silver nanoparticles), and silver ion in the form of $AgNO_3$. The results here evaluated that the best antibacterial efficacy was shown by the synthesized AgNPs as compared to AgNPs, CSNPs, and $AgNO_3$. As compared to synthesized AgNPs (20 nm), the AgNPs have larger size (40 nm) that possess significantly smaller surface area which leads to decreased interaction of bacteria and reduced Ag^+ release. In case of CSNPs, even the smallest size of, i.e., 5 nm, the antibacterial potential is very low. As the CSNPs attributed to a strongly elevated surface tension, the stability of these particles is reduced in biological media and tends to behave like Ag ions. From the previous studies, it was observed that positive charge is an essential condition for the effective antibacterial potential of AgNPs because it facilitates the effective electrostatic interaction of AgNPs to the negatively charged bacterial cell wall. But here the results are showing just contrast where negative charge of the particle contributes more effective antibacterial potential. They suggested that these negatively charged AgNPs represent a safety concern for mammalian cells and tissue by giving a possibility that the cationic NPs show more cytotoxicity toward mammalian cells compared to neutral or negative surface-charged particles. They also experimentally suggested that the Gram-positive bacteria (*S. aureus*), due to the presence of a thicker peptidoglycan cell wall, require tenfold higher concentration as compared to Gram-negative bacteria (*E. coli*) (Salvioni et al. 2017).

Thus, from the studies, it is concluded that the surface charge of the particles rises as the most important factor over other parameters (size and morphology) that confer the antibacterial potential. The electrostatic interaction of negatively charged bacterial cell membrane with positively charged AgNPs leads to effectiveness of antibacterial potential while the negatively charged AgNPs lead to the safety or less cytotoxicity toward the mammalian cell that is a major issue of concern with AgNPs.

6.3 Mechanism of Antimicrobial Effect of Silver Nanoparticles

Silver nanoparticles are well known for their antimicrobial potential which demonstrates greater toxicity toward microorganism as compared to other metallic nanoparticles. These particles are also regarded as the next-generation antibiotics due to their emergence as an alternative to antibiotic therapy. These particles have been evaluated for their antimicrobial potential against a broad range of microbes including bacteria (both Gram-positive and Gram-negative), fungi, and viruses. Further, these particles showed efficacy against various multidrug resistance (MDR) bacteria. Despite ample amount of work being done and further progressively occurring, the precise mechanism of their action of antimicrobial effect is still not completely known (Dakal et al. 2016).

The antibacterial activity of the silver nanoparticles is mainly associated with the generation of silver ions and several evidences are there that suggest that these silver ions Ag^+ are associated with the surface area of the particles. It was evidenced that the higher surface area of the particles is responsible for the release of higher concentration of Ag^+ that leads to increased antimicrobial effect, whereas lower surface area releases lower concentration and ultimately decreased antimicrobial effect (Möhler et al. 2018). Hence, we can say that the antimicrobial property of silver nanoparticles is associated with the quantity and the grade of silver released. Further, the toxicological pathway of silver nanoparticles toward bacteria depends on particles and physicochemical properties. The ample amount of work in this area has shown that silver nanoparticles can disrupt more than one cellular and metabolic pathway of cells for their antibacterial mechanism action (Aziz et al. 2014, 2015, 2016; Abd-Elsalam and Prasad 2018). The mechanism can be broadly categorized into two groups: (1) nonoxidative mechanism and (2) oxidative mechanism (Fig. 6.4).

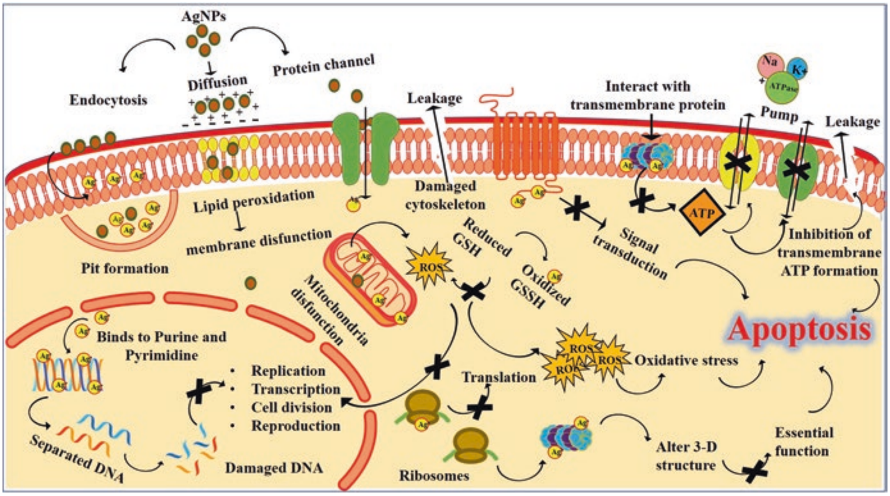

Fig. 6.4 The diagrammatic representation of mechanism antibacterial effect of silver nanoparticles

6.3.1 Nonoxidative Mechanism

The nonoxidative mechanism of antibacterial action of AgNPs includes the anchor of AgNPs to the cell wall generation of Ag^+, alteration of membrane structure, damage of cell wall, penetration into cell membrane, leakage of cellular component, and impaired transport activity.

6.3.1.1 Cell Wall and Cell Membrane Attachment in Bacteria

Silver nanoparticles, when comes in contact with bacterial cells or other microorganisms, the positive charge these particles confer the strong electrostatic interaction to the negative charge on the membrane which facilitate the adhesion or anchoring of these AgNPs to the cell wall. After attachment, some of these particles undergo dissociation into biologically active Ag^+, and further, by Trojan horse mechanism, these ions bind to cell wall and release more Ag^+ into the cell (Singh et al. 2015). These ions further interact with peptidoglycan layer, composed of sugar (β-1,4-linked N-acetylglucosamine and N-acetyl muramic acid) and amine acid (Agnihotri et al. 2014). This interaction leads to the morphological changes which include cytoplasmic shrinkages and detachment of cell membrane. Further, transmission electron microscopy (TEM) studies have revealed the formation of electron-dense pits by cross-linking interaction between sugar and amino acid that leads to site-specific damage of the cell wall. These Ag^+ also interact with sulfur-containing protein in the cell wall that affect the integrity of lipid bilayer and cause permeability. This permeability and pits formation affect the transport activity and disrupt the phosphate ion and potassium ion pump. Ultimately the prolonged permeability leads to the leakage of cellular content out in the surrounding media and causes cell disintegration, and inhibition of cellular function leads to necrosis and cell death.

Further, this nonoxidative mechanism of antibacterial action also depends on bacteria type, whether Gram-positive or Gram-negative. In cash of Gram-positive bacteria, the cell wall is composed of 30 nm thick negatively charged peptidoglycan layer, whereas in Gram-negative bacteria, the layer is of Σ3–4 nm thickness. This structural and compositional difference makes the Gram-negative bacteria more susceptible for the antibacterial action of AgNPs rather than Gram-positive bacteria at same concentration. So, there exists a correlation between the effective concentration of AgNPs and the class of treated bacteria (Patil and Kim 2017).

6.3.1.2 Damaging the Intracellular Structure in Bacteria

After degradation of cell wall of bacteria, this becomes more susceptible for the influx of silver ions by penetration. It was evidenced that the use of surfactant along with these AgNPs can enhance the penetration of AgNPs in the bacterial cells.

After penetration of cell membrane, these particles interact with protein, lipid, DNA, and other biomolecules. Hsueh et al. and Kumar et al. suggested that these AgNPs interact with DNA which causes denaturation and shearing and causes DNA damage. Further, these AgNPs are responsible for the mutation in the essential DNA repair gene of mutant *E. coli*. These particles result in the loss of replicability of the DNA due to change in relaxed to the condensed state. The Ag^+ generated by AgNPs interacts with purine and pyrimidine base separately and destabilize the double helical and β-sheet structure of DNA that leads to inhibition of transcription of gene (Hsueh et al. 2015; Kumar et al. 2016). This leads to inhibition of cell division and their reproduction. The AgNPs also inhibit the process of translation by interacting with the ribosomal subunit and lead to their denaturation. These ions are also responsible for the inhibition of proteins that are involved in the generation transmembrane ATP by reacting with their thiol group. The Ag^+ also interact with the functional group of proteins and inhibit resulted in their deactivation. Further, these ions interact with synthesized protein and inhibit their overall functioning by altering their three-dimensional structure or block their active binding site and leads to overall functional defect in the microorganism.

6.3.2 Oxidative-Stress Mechanism

Oxidative stress is a condition caused by the imbalance in the concentration of reactive oxygen species (ROS) and free radical or we can say increased concentration of these moieties that leads to toxic affect. The AgNPs are responsible for the increased cellular oxidative stress in microbes that is associated with the antibacterial potential of the particles. The electron spin resonance spectroscopy studies evaluated that the increased concentration of Ag^+ generated through AgNPs surface area after interaction with bacterial is expected to cause higher production of ROS and free radical. These ROS and free radical include hydrogen peroxide (H_2O_2), hydroxy radical, superoxide anion (O_2^-), hydroxyl radical (OH), hypochlorous acid (HOCl), and singlet oxygen (Yang et al. 2011).

During normal aerobic respiration, metabolic activity of prokaryotic and eukaryotic mitochondria, chloroplast, peroxisome, and cytosol generates ROS and free radical, and their level is maintained by scavenger like reduced glutathione (GSH). But when AgNPs interact with bacterial cells, the released Ag^+ interact with the thiol group of reduced glutathione (convert reduced GSH to oxidized form glutathione disulfide GSSH) and other related enzymes that leads to disruption of scavenging mechanism (Tang and Zheng 2018). These Ag^+ also bind to the cell membrane of bacteria and cause dysfunction of respiratory electron transporter chain and inhibit respiratory chain enzymes. Further, in the presence of dissolved oxygen, these Ag^+ serve as catalyst for generation of ROS and free radical. This excessive number of ROS and free radical causes damage to mitochondrial membrane, single-strand breakage in DNA components, and necrosis and leads to cell death. Further, hyperoxidation of protein, lipid, DNA, and cell membrane also result in increased ROS (Korshed et al. 2016; Prasad and Swamy 2013; Gupta et al. 2018; Prasad et al. 2017). In case of biologically synthesized AgNPs, the oxidative stress is caused by the elevated level of both ROS and reactive nitrogen intermediate (RNI). Reduction of RNI causes the oxidative stress that is like the effect of antibiotics (Quinteros et al. 2016).

Signal transduction pathway affects the bacterial growth and other molecular and cellular activity. Further, the protein involved in the signal transduction pathway of Gram-negative and Gram-positive bacteria is highly affected by the treatment of AgNPs that leads to inhibit growth and prevent various molecular and cellular activities, and we can say that particles can change the phosphorylation profile of bacterial peptides. So, to examine this offers a useful way to study the toxicity effect or the mechanistic behavior of AgNPs in bacteria (Dakal et al. 2016).

In general mode of action of silver nanoparticles results in:

1. Attachment to the surface of cell membrane
2. Cause structural changes and damage to the cell wall.
3. Pit formation on the site of damage in cell membrane and accumulation of AgNPs occurs on the cell surface.
4. Even short exposure of AgNPs causes accumulation of envelope precursor that leads to degeneration of proton motive force
5. Inhibition of cell wall formation.
6. Prevent the uptake and interchange of phosphate ion that resulted in leakage of membrane for sugar (mannitol), proteins and amino acid (glutamine and proline).
7. Formation of free radical and ROS.
8. Attachment to 30S subunit of ribosome and inhibit the process of translation.
9. The intercalation of bases of DNA leads to hinder the process of DNA transcription.

10. Interact with thiol group of enzymes and protein.
11. Effect on the enzymatic activity of respiratory chain dehydrogenase in bacteria.
12. Inactivate the enzymes involved in the production of ATP.
13. These results in the change of phosphorylation profile that leads to inhibit the signal transduction pathway.

Prevent biofilm formation by arresting the exopolysaccharide synthesis (Agnihotri et al. 2014; Dakal et al. 2016; Hsueh et al. 2015; Korshed et al. 2016; Kumar et al. 2016; Mishra et al. 2018; Möhler et al. 2018; Patil and Kim 2017; Singh et al. 2015; Tang and Zheng 2018; Yang et al. 2011).

6.4 Recent Advancement of Silver Nanoparticles and Their Application

6.4.1 Silver Nanoparticles in Healthcare

6.4.1.1 Wound Healing

The largest human organ, skin, is the first line of defense and acts as a barrier for our body to any infection and damage. But along with this, it is the most exposed part and more prone to any cut and damage that can lead to chronic wound (Nam et al. 2015). These wounds or cuts on the skin are the leading source of bacterial and fungal infection first locally and then spread and become serious infection for the patients. These microbial infections in the wound area have been noted for the delaying in the process of wound healing and sometimes lead to major sepsis, resulting in death of individual (Wilkinson et al. 2011). Further diabetes and obesity patients also suffer with wound, their healing, and sepsis problem. From centuries, silver and its various forms are also known for their tremendous wound healing properties. More specifically green-synthesized silver nanoparticles have been proven to exhibit significant wound healing action (Gong et al. 2018). By taking the advantage of the strong broad-spectrum property of silver nanoparticles, these particles have been extensively being used for treatment of chronic wound healing and their dressing material. These AgNPs have been used as an active agent of large number of dressing material as silver-impregnated bandages and in the form of ointment or cream. These AgNP-coated dressings proved as effective dressing with nontoxicity that can decrease the chance of infection and further accelerate the healing time. Silver nanoparticles are beneficial for wound healing and dressing material due to their characteristic property of broad-spectrum antibacterial activity against MDR and biofilm-forming bacteria along with their anti-inflammatory property that promote wound healing by reducing cytokines release, decreasing mast cell infiltration and lymphocyte. Further these particles can be easily adsorbed on the dressing and cotton fabric that can be applied on the wound area and show very low level of toxicity along with improved mechanical properties (Gong et al. 2018; Gunasekaran et al. 2011).

Guthrie et al. (2012) investigated antibacterial effect of silver-impregnated dressing in vitro and in vivo murine wound infected by *S. aureus*. In this study, author fabricated a silver-impregnated polyelectrolyte multilayers (PEMs) film using poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) (PAA) assembled on poly(dimethylsiloxane) (PDMS). This synthesized Ag-PEMs membrane was then analyzed both in vitro and in vivo for their antibacterial potential. The results of in vitro analysis revealed that the Ag-PEMs significantly reduce the concentration of *S. aureus* (up to 99.99%) as compared to unmodified PEM. Further, the in vivo results revealed that there is absence of any mortality, surgical complication, illness, or weight loss. The Ag-PEM-treated murine wound resulted in reduced bacterial burden in the skin wound as compared to control (Guthrie et al. 2012).

Paladini et al. (2013) synthesized silver nanoparticle-doped hydrogel of diphenylalanine which was modified with aromatic group. These doped hydrogels are proposed to show the antibacterial wound healing potential. So, the experiments are performed against *S. aureus*. The obtained results here suggested that the hydrogel provides the efficient antibacterial potential and moist environment which is required to prevent the infection and promote wound healing, respectively (Paladini et al. 2013).

Anisha et al. (2013) prepared a nanocomposite sponge composed of chitosan, hyaluronic acid, and green-synthesized silver nanoparticles, and the sponge was prepared through freeze-drying method that resulted in a porous structure. This sponge was then evaluated for their antibacterial potential in wound dressing for diabetic foot ulcer (DFU) infection. The analysis was carried out against *E. coli*, *S. aureus*, *methicillin-resistant S. aureus (MRSA)*, *P. aeruginosa*, *and K. pneumonia*. The results here revealed that the nanocomposite sponge showed the effective antibacterial potential against all the test bacteria and the higher concentration of AgNPs (0.005%, 0.01% and 0.02%) containing sponge showed antibacterial potential against MRSA. The results of cytotoxicity study on human dermal fibroblast (HDF) cells showed the concentration-dependent toxicity of AgNPs. In all, the results suggested that the synthesized sponge could be used as a potential material for wound dressing for DFU infected with resistant bacteria (Anisha et al. 2013).

Lin et al. (2014) prepared different concentration of silver containing activated carbon fiber for wound dressing material by using bio-medical carbon technology. After synthesis, this Ag-ACF dressing was evaluated for Ag concentration-dependent antimicrobial activity and wound healing efficacy. The result indicated that the concentration-dependent antimicrobial activity and wound area reduction that is higher concentration lead to effectively prevent the microbial growth and promote the wound healing process. Further, in vivo experiment showed the synthesized Ag-ACF can promote infected wound healing in early phase as compared to the commercial silver dressing used in the same concentration-dependent manner and showed significant biocompatibility (Lin et al. 2014).

Mei et al. (2014) synthesized a cationic polymer poly 2-(dimethylamino) ethyl methacrylate (PDMAEMA-C4) reduced silver nanocomposite which display the enhanced antibacterial potential against both Gram-positive and Gram-negative bacteria due to the synergistic behavior of AgNPs and capped polymer. The results here showed the effect of these synthesized nanocomposite leads to enhanced antibacterial activity against *P. aeruginosa, S. aureus* and improved wound area reduction. Ag@PDMAEMA-C4 also show the efficient antibacterial potential in diabetic rat model and effectively treat the bacterial infected wound (Mei et al. 2014).

Das et al. (2015) prepared an amorphous hydrogel containing in situ synthesized AgNPs, propylene glycol, and carboxymethyl cellulose (CMC) in a single step by heating $AgNO_3$ along with other ingredients. The synthesized hydrogel was then analyzed for their effective antimicrobial activity against MRSA, *S. aureus*, *E. coli*, *and P. aeruginosa*. Further to check the wound healing properties, the in vitro migration or proliferation assay was performed against human dermal fibroblast (HDF) cells. The result showed that the hydrogel effectively reduces the bio-burden of all the test organism including MRSA at 50 ppm concentration. The migration and proliferation results showed the effective cytocompatibility of AgNPs-CMC on HDF cells (Das et al. 2015).

Dai et al. (2016) synthesized ε-polylysine polymer-reduced AgNPs by one-pot synthesis strategy. These EPL-g-butyl@AgNPs were in vitro analyzed for their antibacterial efficacy against *P. aeruginosa and S. aureus*. Further, in vivo antibacterial studies of these synthesized particles were carried on diabetic rat with bacterial infected wound. The results of in vitro assay revealed the effective reduction of growth of bacteria in a dose-dependent manner in case of both bacteria. Further, the maximum ZOI was observed in EPL-g-butyl@AgNPs as compared to AgNPs alone and EPL-g-butyl alone. This was due to the polyvalent interaction and the synergistic effect of both moieties. The results of in vivo experiments showed potential antibacterial effect on the infected wound in diabetic rat model and revealed that the nanocomposite could modulate inflammatory cells thus promoting wound healing without side effect on dermal tissue (Dai et al. 2016).

Tonda-Turo et al. (2016) prepared biodegradable wound dressing using chitosan-based porous membrane via freeze-drying technique which was further impregnated with gentamicin and different ratios of AgNPs (5, 10, and 15%) to improve the antibacterial effect against *E. coli*, *P. aeruginosa*, *P. mirabilis, S. aureus*, and *E. faecalis*. The results here showed the initial burst release of gentamicin on the first day of treatment, but then the decreased release rate was observed that was maintained up to 56 days against bacteria and, hence, can be used as good candidate against reduction of bacterial infection in wound (Tonda-Turo et al. 2016).

Kim et al. (2017a) synthesized a wound dressing material using a cotton wool coated with AgNPs. Here in this experiment, they used solution-dipping method which involves soaking the piece of cotton wool in the alcoholic silver-2 ethylhexyl-carbamates solution (0.01, 0.1, 0.5, 1.0, and 5.0%), followed by thermal reduction at 120 °C. The AgNPs were synthesized on the cotton wool surface in the size range of 20–80 nm and analyzed for their blood clotting property and antibacterial potential against *S. aureus* and *E. coli*. The obtained results showed that the fabric coated with 0.5% AgNPs represents the fastest blood clotting and excellent

UV-blocking in the UV-R range as compared to the unmodified cotton wool. This fast clotting of blood was due to procoagulant activity of AgNPs which increase the concentration of thrombin. Further, the UV-blocking property of the fabric provides protection against intense UV rays. Further, the results of antibacterial assay also represent the maximum antibacterial effect, as shown by 0.5% AgNPs-coated cotton as compared to 0.01, 0.1, and unmodified cotton. All these results go with the essential factors required for wound dressing material (Kim et al. 2017a).

Ahmadi and Adibhesami (2017) performed a study to check the effect of AgNPs on wound contaminated with *P. aeruginosa*. Here the experiment was performed in four groups including negative control (normal saline), positive control (tetracycline), AgNPs alone, and AgNPs + tetracycline treatment. The three groups among them showed effective wound infection treatment where % of wound healing was observed as 98, 99, and 100% in AgNPs, tetracycline, and AgNPs + tetracycline, respectively. Along with this the least bacterial load was observed in case of AgNPs + tetracycline at the site of treatment. These results indicate that the AgNPs and tetracycline show synergistic effect together and the AgNPs are almost equally effective as the antibiotic treatment (Ahmadi and Adibhesami 2017).

Adibhesami et al. (2017) investigated the in vivo effect of AgNPs in wound healing infected with *S. aureus*. The bacterial suspension was added on the back of mouse where a full-thickness skin wound is created. The AgNPs of 20 nm in diameter were used in this experiment. Here the mouse was divided into five groups including negative control (normal saline), positive control (Gentamicin 8 mg/kg), and different concentrations of AgNPs (0.08, 0.04, and 0.02 mg/kg). The results here revealed that AgNPs in 0.08 mg/Kg concentration showed the maximum acceleration in reduction of both wound area and the bacterial load as compared to other AgNPs concentration. On day 21, after treatment the bacterial counts in case of both gentamicin and AGNPs (0.08 mg/kg) were 0.00 CFUg^{-1}, which show no significant difference, whereas, on day 14, the bacterial load of AgNPs was observed as 1.02×10^4 as compared to gentamicin, that is, 3.24×10^4. So, these observations suggested that the AgNPs more effectively reduce the bacterial infections and the wound area in comparison to antibiotics (Adibhesami et al. 2017).

Recently, Liu et al. (2018) prepared a hydrogel for wound dressing that was composed of cross-linked carbohydrate cellulose nanofiber (CNF), gelatin (G), and different concentrations of AgNPs (0.2 and 0.5 mg/ml). The antibacterial efficacy assessment was done via both liquid culture medium and agar disk diffusion. Here also $\text{CNF/G/Ag}_{0.5}$ showed the better performance as compared to only CNF and only AgNPs. Further the results of in vitro and in vivo cytotoxicity and wound healing demonstrate that $\text{CNF/G/Ag}_{0.5}$ showed the maximum $\text{CNF/G/Ag}_{0.5}$ exhibit the maximum biocompatibility, (appr. 100%) cell viability, and wound healing efficiency via efficient growth promoting effect. So, this hydrogel holds the efficient capability of both antibacterial infection and accelerates cell proliferation (Liu et al. 2018).

6.4.1.2 Medical Devices

In the modern era, indwelling devices have become the major and key element in the treatment of various medical conditions. But all these modernizations are somewhere associated with risk of device-associated infections due to bacterial attachment and biofilm formation on the surface of medical devices which can spread the infection to the patients and cause serious sepsis (Bottros and Christo 2014; Smith and Nolan 2013). The AgNPs application, due to their antimicrobial activity, has been increasing in nanoparticle-based medical devices (Aziz et al. 2016). European and international regulator and occupational safety community have raised their vision toward AgNPs due to the pressing need to prevent the increasing healthcare-associated infections (HCAIs) (Marassi et al. 2018).

For this Kim et al. (2017c) developed methods to fabricate the film of silicon, impregnated with silver nanoparticles on a poly-dimethyl-siloxane (PDMS) bed. The AgNPs were then synthesized in situ by reduction of silver ions, and these AgNPs were then grown and allow to electrostatically bind to hydroxyl group formed on the surface of the film after treatment with air plasma. The reduction of $AgNO_3$ occurred through sodium citrate. The film was then assessed for their antimicrobial efficacy against *E. coli* and *B. subtilis*. The results here showed 5 $\log^{10}$ times more reduction of bacterial species as compared to commercial AgNP-mbedded films. So, according to author these films can be useful for the silicon-based implanting on device providing biosafety toward medical device-induced infections and sepsis (Kim et al. 2017c).

Further, Gomez-Carretero et al. (2017) have designed an electro-enhanced antimicrobial coating by using AgNPs. For this, an electrically conducting polymer layer was chemically synthesized using poly(hydroxymethyl 3, 4-ethylenedioxythiophen): polystyrene sulfonate (PEDOT-MeOH:PSS) and 3-(aminopropyl) tri-ethoxy-silane. PEDOT-MeOH:PSS was integrated with AgNPs. The coating was further analyzed for its antimicrobial potential against *S. aureus* (the major colonizing microbe of medical implants) in a comparative manner including electrical dressing and without electrical dressing. From the results it was evaluated that the electro-enhancement (electrical dressing) of the coating membrane showed the complete prevention of biofilm growth (Gomez-Carretero et al. 2017).

Biofilm formation has been found to play role in various infections including catheterized blood stream and urinary tract infection that can lead to major sepsis and mortality. Dybowska-Sarapuk et al. (2017) designed graphene nanolayer for coating of catheters in order to surmount these biofilm infection problems. Here the graphene layer was decorated with AgNPs by spray-coating system. This coating was further assessed for their antibiofilm efficacy against *S. epidermidis* bacteria. It was found that the coated layer of graphene prevents the biofilm completely from the surface of a Foley catheter. Further this method found to be very economic and useful for variety of substrate (Dybowska-Sarapuk et al. 2017).

Apart from antibacterial efficiency, AgNPs can enhance the sensitivity in medical devices. As in the study of Bai et al. (2018), they have designed a surface-enhanced Raman scattering (SERS)-based lateral flow assay strip (LFASs) for the quantitative analysis of biomarkers in low concentration range. Here the authors have enhanced the sensitivity of the probe by using AgNPs along with the widely used AuNPs-LFASs. Here citrate reduction method was used and three forms of citrate capped bio-metallic NPs including Au core with Ag shell NPs (Au@ AgNPs), rattle-like Au core in Ag-Au shell NPs (Au@ Ag-AuNPs) and Ag-AuNPs were synthesized that can be used as optical probe. These synthesized nanocomposites were then evaluated for their solution-based SERS activities. In a detection model, the probe was chosen for the detection of a specific biomarker cTn-I (cardiac troponin-I) for cardiac injury. The results here indicated that the citrate-capped rattle-like Au core in Ag-Au shell NPs (Au@ Ag-AuNPs) exhibit the maximum activity of SERS among all three and were used as both optical and SERS probe in LFASs. The probe was able to detect the signal even at 0.09 ng/ml and that limit can be further lowered by optimization (Bai et al. 2018).

6.4.1.3 AgNPs in Burn Care

The burn injury resulted in the modulation of patient's immune system in which the function of immune cells is suppressed. Further, this leads to increase the susceptibility toward various infections. The mortality rate in case of burn injury patient was mainly due to the sepsis which was due to the massive release of various inflammatory mediators including interleukins (IL-1b, IL-6, IL-8, IL 10), inflammatory cytokines, and interferon-gamma (INF-γ). Further, it was observed the IL-10 deficiency or aberrant expression leads to inflammatory bowel disease and several autoimmune disease (Shaheen 2016). Silver nanoparticles, due to their effective antibacterial, anti-inflammatory, and angiogenic properties, have potential application in the treatment of burn care injuries and wounds (Gurunathan et al. 2009; Nadworny et al. 2008).

Muniyan et al. (2017) synthesized AgNPs by using a green eco-friendly manner which showed the effective antibacterial potential against bacterial species related to severe burn wound infection including *E. faecalis*, *S. aureus*, *A. baumannii*, *E. asburiae*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, and *P. aeruginosa*. For the synthesis purpose, they use fenugreek seed as a source of saponins and used for the reduction of $AgNO_3$ to produce saponin-capped AgNPs (SN-AgNPs). The results of both disk diffusion assay and MIC revealed that the synthesized SN-AgNPs efficiently show their antibacterial potential against all test organisms. The enhanced potential was due to the synergistic effect of saponin (that can cause destabilization of the permeability of bacterial membrane) and AgNPs (Muniyan et al. 2017).

Luna-Hernandez et al. (2017) synthesized chitosan and silver NPs composed film with ultralow silver level and without aggregation of AgNPs for dressing of thermal burn wounds. The application of this nanocomposite film in the treatment of burn wound resulted in the higher antibacterial potency even at 10 nm concentration of AgNPs embedded in the film against *S. aureus* and *P. aeruginosa* infection. The healing of wound is also observed by inducing increased myofibroblast, collagen remolding, new blood vessel formation, and regeneration of epiderma after

7 days of treatment. Further, these synthesized nanocomposites are able to show biocompatibility and, hence, can be a potent material for burn wound care (Luna-Hernandez et al. 2017).

Stojkovska et al. (2018) prepared alginate and silver nanoparticle-based nanocomposites for the treatment of wounds that were due to second-degree thermal burn in model Wistar rat. Here electrochemical method was firstly used to synthesize AgNPs, which were then mixed with alginate solution to obtain microfibers for in vivo application. The infected rats were then divided into five groups for different levels of treatment to perform a comparative study. The groups include G1 (untreated wound), G2 (commercial cream containing silver sulfadiazine), G3 (AgNPs/alginate and ascorbic acid), G4 (Ca-alginate containing Ag^+), G5 (wet AgNPs/alginate microfiber), and G6 (dry AgNPs/alginate microfiber). The in vivo experiment results revealed that the maximum wound healing was observed in group G4, G5, and G6 in a shortest period of 19 days. Whereas G2 and G3 showed significant wound healing after 21 days and G1, the untreated wound takes 25 days. Further, the histopathological studies showed the results of enhanced granulation and re-epithelialization in the same manner. There results have shown the effective potential of the synthesized nanocomposites based on alginate and AgNPs for burn wound treatment (Stojkovska et al. 2018).

6.4.1.4 Bone Cement

Bone is complex structure which is composed of living cells embedded in a biomineral matrix (consisting 30% organic and 70% inorganic material). The solid bone is formed by hardening of this biomineral matrix around the entrapped cells. The orthopedic extremity trauma leads to complication of larger segment bony defect and nonunion (Kanakaris and Giannoudis 2007). Further the tissue barrier loss between the site of fracture and external environment leads to the susceptibility of bacterial infection and sepsis. For larger segment loss of bone, the conventional treatment and repair method includes use of polymeric cement spacer. The spacer embedded with antibiotics is generally nondegradable. These conventional methods are associated with limitation as (1) the embedded antibiotic effect locally due to poor release and rise in antibiotic resistance. (2) The spacer removal by second operation and (3) the cement spacer can cause bacterial biofilm infection (Behzadi et al. 2017).

Nanotechnology application, by designing and engineering of nanomaterials, opens a new way that can surmount the issue of conventional therapies (Farokhzad and Langer 2006). Nanoparticles incorporation gives an alternative to antibiotics that can resolve the multidrug resistance issues (Guzman et al. 2012; Montanaro et al. 2011). Among metal, silver nanoparticles are best suited for this purpose due to their anti-inflammatory, antiangiogenic, and most importantly antibiotic and antibiofilm activity. An ample amount of work has been progressing in this area (Gurunathan et al. 2009; Nadworny et al. 2008).

González-Sánchez et al. (2015) synthesized methacrylate hydrogel for artificial bone graft application with incorporation of silver nanoparticles in it. In their work, they first synthesized silver nanoparticles using sodium citrate reduction, and these particles were then integrated into the hydrogel at two different (0.5 and 1 mM) concentrations. The integration was done by using three different methods including entrapment of AgNPs before mineralization, diffusion, and adsorption after mineralization. The synthesized hydrogels were then assessed for their antimicrobial potential against *S. epidermis* and methicillin-resistant *S. aureus* (MRSA). The results revealed that among the three cross-linking methods, only adsorption of AgNPs on biomineralized hydrogel represents the antibacterial efficiency. Further the potential was independent of the concentration of AgNPs cross-linking. The cytotoxicity results on osteoblast (MC-3T3) cells represent the nontoxicity, and no detrimental consequence was observed on rheological characterization (González-Sánchez et al. 2015).

Zhang et al. (2016) modified alginate with mussel-inspired dopamine to prepare a gel which was coated by AgNPs. The gel was expected to promote the osteogenic differentiation of mesenchymal cells and reduce the bacterial infections. The comparative study was performed using alginate-dopamine and alginate-dopamine-AgNPs beads and fibers. The resulted gel showed efficient antibacterial potential against *S. aureus* and *E. coli*; the gel also showed the excellent cell viability and bone regeneration activity even in contaminated bone (Zhang et al. 2016).

Zhang et al. (2017) prepared bio-ceramic scaffold using β-tricalcium phosphate (β-TCP) which was further modified with silver and graphene oxide nanoparticles (Ag@GO) via liquid chemical reduction approach. The nanocomposites were then prepared in different Ag to GO mass ratio. The antibacterial potential was assessed against *E. coli* where the results revealed that the β-TCP scaffold modified Ag@GO does not show effective antibacterial activity but positively promotes the expression of osteoblast-related gene. Further, nanocomposite of Ag and GO showed reduction in bacterial growth (Zhang et al. 2017).

Further, a novel 3D printed polyetheretherketone (PEEK) has been developed by Deng et al. (2017) via catecholamine chemistry which was surface decorated with AgNPs. For in vitro antimicrobial and antibiofilm analysis, comparative study between 3D PEEK and 3D PEEK/Ag (1 mM) was performed against *E. coli* and *S. aureus*. The results here showed that the 3D PEEK/Ag (1 mM) showed the efficient contact killing and release killing. Further, it was effective for the rupture of biofilm. The in vitro studies of MG-63 cells, via LDH assay and ALP test, also revealed 3D PEEK/Ag (1 mM) exhibit maximum activity in both experiment and is proven as potential scaffold for osteoblastic differentiation (Deng et al. 2017).

Aurore et al. (2018) performed their study to evaluate the effect of silver nanoparticles against the osteoclast infection by *S. aureus* and *E. coli*. Here the study revealed the mechanistic behavior of antibacterial effect of AgNPs toward bacteria in which ROS moiety increased that is responsible for reduction of bio-burden of *S. aureus* and *E. coli* (Aurore et al. 2018).

Further to achieve osteoinductivity along with antibacterial properties, Mao et al. recently (in 2018) prepared microsphere (PM) of strontium ranelate (SR)-loaded poly(lactic-co-glycolic acid) (PLGA) which was assembled with AgNPs and hydroxyapatite nanoparticles (HANPs). The SR-PM-Ag-HA microsphere was prepared using solid-in-oil nanosuspension method. The in vitro studies were conducted in a comparative manner between PM, SR-PM, SR-PM-Ag, and SR-PM-Ag-HA. The results revealed that all the synthesized microspheres are biocompatible against MC3T3-E1 cells, and except PM, all other form of microsphere showed adequate drug release efficiency along with effective proliferation. The best osteogenic differentiation of MC3T3-E1 cells was exhibited by SR-PM-Ag-HA. Further the antibacterial analysis and antibiofilm assay against *S. epidermis* and MRSA bacteria revealed that SR-PM-Ag-HA and SR-PM-Ag showed effective potential in both bacteria (Mao et al. 2018).

6.4.1.5 Dental Care

Silver in oral care, known from centuries, gaining worldwide spread as the main component in dental amalgams (Rueggeberg 2002). Silver nanoparticles due to their effective antimicrobial properties have been extensively used in dental restoration materials that show efficiency against the bacteria producing carries, i.e., *Streptococci* and *Lactobacillus* (Melo et al. 2013). Along with its antibacterial potential these particles effectively provide strength to the incorporated self-etching materials that can further help in restoration dentistry (Fatemeh et al. 2017).

Fan et al. in 2014 conducted study to evaluate the effect of silver nanoparticles in the growth inhibition of bacterial colonization of dentine. Here, they used mesoporous calcium-silicate nanoparticles (MCSN). The synthesized silver nanoparticles were then incorporated in the biomaterial by two methods including adsorption and template method. These biomaterials were then tested against *E. faecalis* in different forms (MCSN alone, AgNPs adsorbed MCSN, and AgNPs templated MCSN) to evaluate their antibacterial potential. Here, the results revealed that both surface-adsorbed and surface-templated AgNPs-MCSN significantly inhibit the bacterial attachment and colonization of dentine. Further, as compared to adsorption AgNPs, templated AgNPs-MCSN were much lower in cytotoxicity and resulted in controlled release of Ag^+ that can inhibit both planktonic and biofilm that can be effectively used for root canal disinfection (Fan et al. 2014).

Emmanuel et al. (2015) used an eco-friendly approach for the systemic of AgNPs when the *Justicia glauca* leaf extract was used as reducing agent. The synthesized particles were in the range of 10–20 nm. These particles were then tested for their antimicrobial potential against *S. mutans*, *S. aureus*, and *Lactobacillus acidophilus*. The results here evaluated that AgNPs showed effective antimicrobial effect in a dose-dependent manner (Emmanuel et al. 2015).

Inbakandan et al. (2016) also synthesized flower-like silver nano-colloids using a green biosynthesized method. Here *Haliclona exigua* (marine sponge) extract was prepared used for the reduction of silver salt to AgNPs. These nanoparticles were then used against the oral biofilm-forming bacteria including *S. orulis, S. salivarius*, and *S. mitis*. The results showed that even at 10 μg, concentration of AgNPs can effectively inhibit the growth of these test bacteria (Inbakandan et al. 2016).

Further, Fatemeh et al. (2017) performed a study to evaluate the effect of silver nanoparticles on the composite shear bond strength to the exposed dentine. Here firstly the AgNPs were prepared using sodium dodecyl-sulphate (SDS) that also act as capping agent for these particles. Further specimen preparation was done by using six different types of adhesive system and AgNPs. Here the results revealed that the AgNPs can improve the bonding effectiveness of Clearfil SE bonds on the wall of cavity. Further, it was evaluated that the bond strength of etch-and-rinse adhesive was effectively high as compared to self-etch (Clearfil SE bond) adhesive. So this study revealed the effective antibacterial potential in restorative dentistry and can increase the bond strength of adhesive system (Fatemeh et al. 2017).

6.4.2 Silver Nanoparticles in Textile

Nowadays, due to immense advancement of nanotechnology, researchers are focused in the field of textile industries where ample amount of work is going on the fabrication of antibacterial, self-cleaning, breathable, UV protectant, dirt, and water-repellent fabric. The objective of this advancement is there to fulfill the complete bactericidal potency of fabric that can be used in undergarments, sportswear, hospital clothing, work-wear, seat, and carpets of vehicles that can improve the quality of life by control of spreading microorganism from one person to other. Several studies are there with the use of silver nanoparticle-embedded fabric.

Pulit-Prociak et al. (2016) used silver nanoparticle-modified dye which can be used for dyeing cotton fabric that gives permanent antimicrobial property to fabric. Here firstly, dyes (indigo and yellow commercial dye) were prepared and AgNPs were reduced in situ with dye. The silver-modified dyes were then used for coloring the cotton fabric which was then assessed against *S. cerevisiae* strain to evaluate their antibacterial potential. Results indicated that these fabric showed efficient antibacterial potential along with UV-protection property (Pulit-Prociak et al. 2016).

Ballottin et al. (2017) synthesized silver nanoparticles from a biogenic method where *Fusarium oxysporum* fungal filtrate (FF) solution was used for the reduction of silver to $AgNP_1$ and $AgNP_2$. These AgNPs were impregnated on cotton fiber that can be used in medical and agricultural clothing to avoid the spread of microbes and severe infection. The antimicrobial potential of these fabricated cotton was evaluated against fungi (*Candida albican, Candida parapsilosis*) and bacteria *Xanthomonas axonopodis pv. citri*. The cytotoxicity study was evaluated against standard fibroblast cell line 3T3. The evaluated results showed that $AgNP_2$ exhibit efficient effectiveness with MIC value lower as compared to $AgNP_1$ due to high Ag^+ release in case of $AgNP_2$ (Ballottin et al. 2017).

Recently, Li et al. in 2017 developed a method for preparation of cotton fabric with characteristic antimicrobial and UV-protection properties. In their study, they used hydrothermal deposition method for deposition of flower-like TiO_2

micro-particles onto the cotton fabric. The AgNPs were then in situ synthesized onto this TiO_2@ cotton surface that was pretreated by sodium hydroxide solution. The AgNPs were deposited in different concentration to check their comparative concentration-dependent effect against bacteria (*S. aureus and E. coli*) and UV. The comparative study was done between AgNPs@ cotton, TiO_2@ cotton, and Ag/TiO_2@ cotton. The results of in vitro assay revealed the good antibacterial efficacy of both AgNPs@ cotton and TiO_2@ cotton, while the maximum antibacterial potential and high UV-protection factor of 56.39 was observed in Ag/TiO_2@ cotton which was due to the synergistic behavior of both TiO_2 and AgNPs (Li et al. 2017).

6.4.3 Silver Nanoparticles in Water Treatment

Conventional water purification and treatment methods for disinfection occur through ozone and chlorine-based treatment that are associated with the limitation due to generation of various by-products. AgNPs, due to their characteristic antibacterial potential, have ability to avoid or surmount these issues (Gadkari et al. 2018).

In 2014, Adibzadeh et al. fabricated nanofibrous membrane via electrospinning for disinfection of contaminated water. Here the membrane was fabricated using chitosan and polyvinyl alcohol with 70 to 30 mass ratio, respectively. Further, the silver nanoparticles were decorated on the surface of membrane by in situ reduction of $AgNO_3$. To evaluate the disinfectant potency, the membrane was assessed against *E. coli* that resulted in efficient reduction in bacterial growth and exhibit significant increase in both static and dynamic antibacterial filtration efficiency (Adibzadeh et al. 2014).

Qurban Ali et al. in 2017 fabricated multifunctional nanocomposite that is composed of multiwalled carbon nanotubes (MWCNTs). These nanocomposites were then embedded with iron oxide and AgNPs. Due to antimicrobial potency of embedded AgNPs, this nanocomposite was used to treat the *E. coli*-infected drinking water (Ali et al. 2017).

Recently, Elwakeel et al. in 2018 synthesized magnetic disinfectant through the polymerization of thiourea and formaldehyde in the presence of magnetite nanoparticles (MTUF). The obtained polymerized magnetic chelating resin was then impregnated with Ag ions. Further, to evaluate the antibacterial potential, the resultant MTUF-Ag was assessed against Gram-negative bacteria including *E. coli*, *S. typhimurium*, *P. aeruginosa*, and Gram-positive bacteria including *Listeria monocytogenes*, *S. aureus*, *E. faecalis*, and *B. subtilis*. The results of in vitro study revealed that the minimum inhibitory dosage (MID) after 40 min of treatment of MTF-Ag was 1.5, 2.0, 1.0, 1.5, and 1.5 mg/ml for *E. coli*, *S. typhimurium*, *L. monocytogenes*, and *S. aureus*, respectively, whereas the MID value 2.5 mg/ml in *C. albicans* revealed that the bacteria have resistance potential against this magnetic disinfectant (Elwakeel et al. 2018). All these studies revealed that the AgNPs can be successfully used as a good candidate for water disinfectant.

6.5 Possible Challenges for Silver Nanoparticles

The resistance toward antibiotics among bacteria has now became a major concern in environment and public health. Further, emergence of superbacteria that are resistant to almost all antibiotics are worsening the situation. There are two aspects of occurrence of antibacterial resistance through mutation in their genome or acquisition of resistance gene by horizontal gene transfer (Hocquet et al. 2016). So, to combat with these conditions, silver nanoparticles were focused by researcher, but the expected risk of repeated intentional (as biocide) and unintentional (dispose-off of various commercial consumer products) use of these AgNPs can lead to major resistance problem. Although there are no clear reports on bacterial resistance toward AgNPs, there is presence of resistance reports toward Ag^+ (von Wintersdorff et al. 2016).

Hachicho et al. in 2014 presented a study where they evaluated the effect of AgNPs and Ag^+ on the adaptive response mechanism and growth of *Pseudomonas putida mt-2* (the best strain for stress response investigation). Their investigated results show that the Ag^+ was about 1600 times more toxic than AgNPs and the activity of these AgNPs reduced with the decreased amount of silver ion release. Further, from the experiment they found that the *P. putida mt-2* excluded the toxicity effect of $AgNO_3$ and AgNPs. It was reported in the literature that the *Pseudomonas* strain have ability to modify the surface hydrophobicity of their cell envelope. So, here author suggested that it could be a reason of excluded toxic of $AgNO_3$ and AgNPs (Hachicho et al. 2014).

Further in 2015, Graves et al. performed a study where they utilized citrate-coated AgNPs and $AgNO_3$ against *E. coli* K-12 MG1655 (not known for any silver resistance element). In their experiment *E. coli* was exposed to AgNPs environment for a larger number of generation. The results of genomic analysis revealed that a total of three mutations were observed to occur after treatment of AgNPs up to 200 generations, which leads to high-frequency resistance toward these particles. Finally, the exposure of AgNPs upto 225 generations resulted in a complete resistance in the presence of 10 nm concentration of citrate-capped AgNPs and $AgNO_3$ (Graves Jr et al. 2015).

Kaweeteerawat et al. (2017) performed study of AgNPs resistant effect and mechanism against *S. aureus* and *E. coli*. Here the results showed, after treatment of AgNPs the bacteria showed 3–13 fold more resistance toward ampicillin and pen-step. Further, in case of kanamycin, chloramphenicol, and penicillin, the MIC and MBC value increased up to eight fold. This occurred by lowering of both the membrane damage and oxidative stress. Further, it was observed that the repeated penetration of AgNPs resulted in promoting stress tolerance generated through ROS and free radicals (Kaweeteerawat et al. 2017).

These resistance issues can be resolved by the combinational use of silver with antibiotics where synergistic effect of antimicrobial agent reduces the chance of microbial resistance and occurs due to single mechanism of resistance (Graves et al. 2017).

Recently, Panacek et al. in 2018 have performed experiments against Gram-negative bacteria *E. coli* 013, *P. aeruginosa* CCM 3955, and *E. coli* CCM 3954 to evaluate the mechanism behind the development of bacterial resistance toward AgNPs. Their results also revealed that the resistance of bacteria toward AgNPs can be developed by repeatedly exposing to inhibitory concentration. They also revealed the mechanism of bacterial resistance is due to the production of adhesive protein (flagellin) in the bacterial flagella which enhance aggregation. They also revealed that the problem can be resolved by inhibiting the flagellin production that will reduce the aggregation of particles toward flagella. Further their study revealed that the Pomegranate rind extract (PGRE) can be used against flagellin to inhibit production of flagella. This can minimize the existing challenges toward the use of silver nanoparticles against bacteria (Panáček et al. 2018).

6.6 Conclusion and Future Prospects

Regardless of the preparation methods, all fabricated AgNPs can exhibit their antimicrobial potential in a good manner. This potential further depends on the various physicochemical properties including size, shape, state, charge, concentration, and dose of the particles. The efficient antibacterial potential of these AgNPs can make it applicable in broad-spectrum area such as healthcare, medical devices, cosmetic, food and beverage packing, purification units, and sterilized coating materials. These particles are emerging as a powerful weapon against various multidrug resistance bacteria. Further, these particles efficiently show their potential toward biofilm-forming bacteria and reducing the bio-burden in healthcare and medical field. Nowadays, researchers are extensively trying to focus on these AgNPs for their antibacterial potential against superbacteria that is giving a hope to minimize the worsening of condition of present scenario. Despite all these spectacular benefits, there are emergence of new reports of antibacterial resistance toward Ag^+ released through AgNPs which have been observed due to the intentional (as biocidal) and unintentional (other consumer products) exposure of these AgNPs toward bacteria in an extensively repeated manner. So, all these findings indicate that care should be taken with regard to the use of unintentional exposure of microbial communities to these AgNPs in waste products.

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Chapter 7
Recent Progress on Novel Ag–TiO$_2$ Nanocomposites for Antibacterial Applications

Jai Prakash, B. S. Kaith, Shuhui Sun, Stefano Bellucci, and Hendrik C. Swart

7.1 Introduction

Bacterial disinfection is one of the basic needs for a healthy society, as many diseases are originated from microbes (Deshmukh et al. 2018). Therefore, the scientific community is working to produce new and efficient antimicrobial materials to effectively protect the human body without affecting human lives. Various nanomaterials have been investigated for antibacterial applications, including polymers, metals, and metal oxide photocatalysts and their nanocomposites (Prakash et al. 2018), and this field is growing in search of very effective antimicrobial materials with superior antimicrobial properties under various circumstances (Hoseinnejad et al. 2018; Mukherjee and De 2018).

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Photocatalyst-based nanomaterials have emerged as novel multifunctional materials for multidisciplinary research and applications (Prakash et al. 2018; Singh et al. 2017a, b; Jai et al. 2016). In the field of antibacterial research particularly, photocatalysts such as titanium dioxide (TiO_2)-based nanomaterials have been designed and engineered to enhance their photocatalytic antibacterial functionality (Jai et al. 2016; Prakash et al. 2018). The engineering and tuning of their physicochemical properties enhance their optical properties, including enhanced rate of separation of charge carriers, charge transportation that eventually enhances photocatalytic antibacterial efficiencies (Prakash et al. 2018).

TiO_2 is a material well known for its photocatalytic activity. Since the discovery of its photocatalytic activity in 1972 by Fujishima and Honda (1972), TiO_2 has been used extensively as a photocatalyst material in a variety of fields and applications. It was found that under UV light irradiation, holes (h^+) and electrons (e^-) generated in the valence band (VB) and conduction band (CB), respectively, undergo surface reaction with atmospheric oxygen (O_2) or water (H_2O) molecules, leading to the formation of reactive oxygen species (ROS) (Prakash et al. 2018). The ROS thus generated on the surface of TiO_2 participate in photocatalytic degradation of various organic compounds (Singh et al. 2017a, b). Similarly, TiO_2 exhibits antibacterial activity, resulting in deactivation of bacteria (Jai et al. 2016; Prakash et al. 2018), which was first investigated by Matsunga et al. (1985) in the 1980s. Thereafter, TiO_2 has been extensively used as an antibacterial agent with potent killing ability for several kind of microorganisms under the influence of UV irradiation (Matsunaga et al. 1985, 1988; Prakash et al. 2018; Jai et al. 2016; Maness et al. 1999). In spite of its great potential to be used as an antibacterial or photocatalytic material, TiO_2 has the drawback of fast recombination of UV-generated e^- and h^+ pairs, inhibiting its functionality (Prakash et al. 2018). Also, TiO_2 is a wide bandgap material, which inhibits its potential application in extended visible light of the solar spectrum. However, TiO_2 is the most widely used antibacterial material for reasons of its higher chemical stability, lower production cost, and better availability. It has been of great interest among researchers to improve the antibacterial functionality of TiO_2 materials during the past few decades, and this is still a growing research area with the development of new materials and methodologies (Prakash et al. 2018; Deshmukh et al. 2018).

The antibacterial activity of TiO_2 has been found to be improved by assembling with functional nanomaterials, which also facilitates the charge separation and extends their utilization in visible light for maximizing its practical applications in a wide range. Among various functional nanomaterials, silver nanoparticles (Ag NPs) are widely known as an antibacterial material and have been extensively used in the biomedical field for killing bacteria (Jalali et al. 2016; Yang et al. 2016; Kędziora et al. 2018; Aziz et al. 2014, 2015, 2016, 2019). As compared to TiO_2, Ag shows antibacterial activity without any light activation. In addition, a nanocomposite of TiO_2-based nanomaterials with Ag NPs not only enhances the antibacterial activity under UV irradiation but also extends up to visible light-induced antibacterial activity through formation of a Schottky junction at the interface and surface plasmon resonance (SPR) properties, respectively (Prakash et al. 2018). These nanocomposites have been studied in a broad range of antibacterial activities for reasons of the better electron transfer properties (charge separation) and visible light-activated antibacterial activities (Jai et al. 2016). For example, Quiñones-Jurado et al. (2014) reported that Ag NP-assisted visible light activity enhanced the antibacterial effect of TiO_2 because of the SPR properties, whereas TiO_2 itself did not show any activity under visible light. The nanocomposites of Ag and TiO_2 have shown complementary enhanced antibacterial effects for both the Ag and TiO_2 nanomaterials. For example, Yaşa et al. (2012) studied the antibacterial effect of Ag NPs and Ag-embedded TiO_2 nanocomposites. It was found that the nanocomposite exhibited greater antibacterial effect with an inhibition zone of 12–13 nm as compared to the inhibition zone of 8–9 nm of Ag NPs. Similarly, Mai et al. (2010) developed Ag–TiO_2 nanocomposites-based antibacterial coating and found that the positive antibacterial activity of TiO_2 showed an enhanced antibacterial effect of Ag as a result of galvanic effect (Cao et al. 2011). Several other factors affect the antibacterial effect of these nanocomposite materials, such as the shape, size, and concentration of the components in the composite. For example, Zhang and Chen (2009) studied the effect of size of Ag NPs in Ag–TiO_2 nanocomposites on their antibacterial activity. Ag NPs of various sizes were produced in Ag–TiO_2 nanocomposites, and Ag NPs of less than 3 nm showed complete inhibition in bacterial growth of *Escherichia coli*, attributed to the small size of the Ag NPs and the unique structure of the TiO_2 NPs. Deshmukh et al. (2018) studied the effect of concentration of Ag NPs anchored on TiO_2 nanocrystals for antibacterial activity under visible and UV light irradiation against *Staphylococcus aureus* and *E. coli* bacterial strains, reporting excellent antibacterial effect along with the dynamics of their antibacterial action. Similarly, Li et al. (2017a) demonstrated higher antibacterial effect with inhibition rate of more than 99% against these bacterial cells using Ag NPs-loaded TiO_2 cotton. Jalali et al. (2016) investigated antibacterial functionality of Ag NPs, Ag–TiO_2 NPs, and also NPs immobilized in a silane sol-gel matrix. The Ag–TiO_2 NPs exhibited better antibacterial response, as compared to Ag NPs only, against Gram-negative rather than Gram-positive bacteria. The Ag–TiO_2 NPs showed excellent antibacterial performance even when embedded in a silane matrix for a longer period, attributed to the slower release of Ag^+ from this nanocomposite.

These results show that the antibacterial materials based on the Ag–TiO_2 system could be very efficient for antibacterial activities because of the enhanced optoelectronic properties in the nanocomposite form. In this chapter, we review the latest advances in this field with emphasis on the mechanisms of their antibacterial action and details of various factors influencing their antibacterial activities.

7.2 TiO_2 and Its Antibacterial Activity

As we discussed in the previous section, TiO_2 is widely known for its photocatalytic activities along with antibacterial applications under the excitation of UV light because of its wide bandgap. Under the influence of UV light irradiation, e^- and h^+

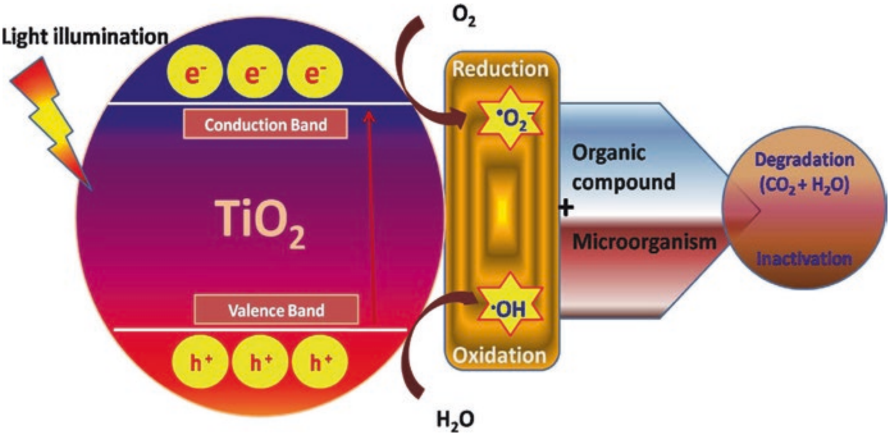

Fig. 7.1 Schematic showing photocatalytic action of TiO_2 nanomaterials under UV irradiation. As a result of illumination, reactive oxygen species (ROS) (•O_2–,•OH, H_2O_2) are generated and react with organic molecules and microorganisms on the surface of TiO_2 nanoparticles (NPs). (With permission from Prakash et al. 2018)

are generated in CB and VB, respectively, which react with water and oxygen molecules, leading to the formation of ROS (Fig. 7.1) (Prakash et al. 2018). These ROS, produced as a result of photocatalytic reactions, exhibit strong oxidizing behavior towards organic molecules, showing strong photodegradation activities (Prakash et al. 2018).

Similarly, when these ROS interact with bacteria or any other living organisms, the cell membranes of the bacteria or living organisms are damaged, causing the death of the cell (Prakash et al. 2018). Several ROS such as ·OH and ·O_2 are produced on the surface of TiO_2. However, ·OH is produced in the majority that effectively takes part in the photocatalytic activity of photodegradation and antibacterial effect (Joost et al. 2015). When interacting with the bacterial surface, TiO_2 causes photooxidation of the cellular components such as oxidation of plasma membrane phospholipids (Maness et al. 1999; Kiwi and Nadtochenko 2004; Joost et al. 2015). The antibacterial mechanisms of TiO_2 nanomaterials have been extensively investigated, suggesting that during antibacterial action first ROS damage the cell membrane, leading to leaking of the cellular components and oxidation, followed by complete inactivation of the cell as shown in the schematic of Fig. 7.2 (Liou and Chang 2012).

Maness et al. (1999) showed inactivation of *E. coli* bacterial cells under UV irradiation of TiO_2 as a result of lipid peroxidation of phospholipid within the cell membrane through ROS attack, leading to damaging of the cell respiratory system; eventually the cells died. Similarly, damage of DNA caused by the formation of H_2O_2 ROS under UV irradiation of TiO_2 material has been reported to be responsible for cell death (Hirakawa et al. 2004). Similarly, Sunada et al. (2003) proposed the photokilling antibacterial action mechanism of TiO_2 for *E. coli* bacteria with similar conditions and explained the process of the photokilling mechanism based

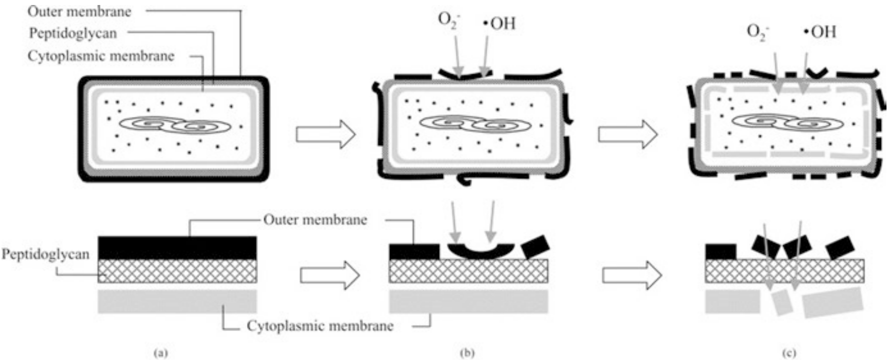

Fig. 7.2 (**a**–**c**) Schematic photokilling action mechanism in *Escherichia coli* on TiO_2 film. For clarity, the part of cell membrane affected by photoirradiation has been magnified. (With permission from Sunada et al. 2003)

on the structural differences of the cell walls in the bacteria. However, it was found that the excellent antibacterial activities of TiO_2-based nanomaterial were reported mainly under the influence of UV light irradiation, and no more, or poor, antibacterial effects were observed with irradiation of longer wavelength (Prakash et al. 2018; Joost et al. 2015) because of the wide bandgap of TiO_2 (bandgap is 3.03 and 3.20 eV for rutile and anatase phases, respectively) (Prakash et al. 2018). Various methods have been used to improve the antibacterial activity of TiO_2 under UV light irradiation and extend its antibacterial capability into the visible light region for maximum applicability. Recently, Guo et al. (2017) demonstrated that annealing of TiO_2 in the presence of metals introduced some defects that were attributed to the antibacterial activity, and also showed visible light absorbance without production of any ROS. On the other hand, Liu et al. proposed that the formation of a surface hetero-junction of different facets on polycrystalline TiO_2 promoted better charge separation and exhibited excellent antibacterial activities through ROS generation on the surface, leading to the inactivation of microbes (Liu et al. 2017). The formation of nanocomposites with Ag NPs is one of the best strategies for improving the antibacterial activity of TiO_2 because Ag NPs also show antibacterial effect as well as extending the applicability of TiO_2 into visible light (as discussed in the next sections).

7.3 Ag NPs and Their Antibacterial Activity

Metallic NPs are interesting nanomaterials to the scientific community as their properties at nanoscale can be tuned by engineering their shape and size, with potential utilization in several industries including biotechnology. These NPs have also been used in antibacterial and other medical applications (Patil and Kim 2017; Prakash et al. 2018). Recent developments in the biomedical field show that NPs are being used in many medical treatments to provide a better platform for fighting several diseases (Prakash et al. 2018). Particularly, Ag NPs have been widely used for their excellent antibacterial action towards various bacteria and other microorganisms because of their large surface-to-volume ratio and their unique physiochemical properties and toxic nature (Prakash et al. 2018; Aziz et al. 2016; Prasad 2014). Extensive research has been carried out on the antibacterial action of Ag NPs but the exact mechanism of this antibacterial action is not very clear, with different researchers holding different views. In some cases, the mechanism of antibacterial action depends on the kind of bacteria, their size, or the concentration of the Ag NPs. It has been reported that smaller Ag NPs can make sufficient contact on the bacterial cell wall, resulting in better antibacterial action. However, all bacteria are not the same and differ in their chemical composition and structure, thus showing a different response when contacting Ag NPs. For example, the antibacterial action mechanism of Ag NPs towards Gram-positive and Gram-negative bacteria is different because of their different cell wall structure and composition. Gram-positive bacteria have a thick cell wall as compared to Gram-negative bacteria, with many layers of peptidoglycan polymer (Prakash et al. 2018). This structural difference in the cell membrane of these bacteria affects the permeability of Ag NPs or Ag ions for further antibacterial action. The mechanism also depends on the type of interaction when Ag NPs come into contact with the bacterial cell wall. For instance, Ag^+ interacts more efficiently with Gram-negative bacteria, changing the cell membrane by leaking into the cell wall and thus affecting the respiratory activities of the cell. Particularly, Ag NPs interact with phosphorus and sulfur in the bacterial cell membrane and other intercellular components, such as DNA bases and proteins, affecting the various bacterial activities and leading to its death (Joshi et al. 2018; Prasad and Swamy 2013). The various mechanisms of antibacterial action of Ag NPs and Ag^+ are shown in the schematic of Fig. 7.3 (Patil and Kim 2017). In a recent article, Verma et al. (2018) reported the antibacterial activities at molecular level with a detailed mechanism of the in vitro cytotoxicity effect of green synthesized Ag NPs against various cells. They demonstrated that the cytotoxicity effect was attributed to the variable interaction of the cell components with Ag NPs, followed by oxidative stress leading to cell death (Aziz et al. 2019). Similarly, Prema et al. (2017) reported that reductive decomposition of various cell components caused by the interaction of Ag NPs led to death of the cells.

The unique and fascinating electrical, optical, physical, and chemical properties of these NPs has led to their utilization in a wide range of applications such as catalysis, sensing, environmental, and biomedical uses, including antibacterial actions (Prasad et al. 2017, 2018). For example, noble metal NPs such as Ag and Au NPs are interesting nanomaterials that gained the attention of researchers of all fields, as already mentioned, because of their unique physiochemical and optoelectronic properties. These unique properties result from their SPR properties, which arise from the collective oscillation of free electrons on their surface (Fig. 7.4) (Singh et al. 2017a). The optical properties of these NPs can be refined by tuning their SPR properties by engineering their shape, size, and surrounding environment. Effects of these factors on SPR properties of noble metal NPs are reported in many

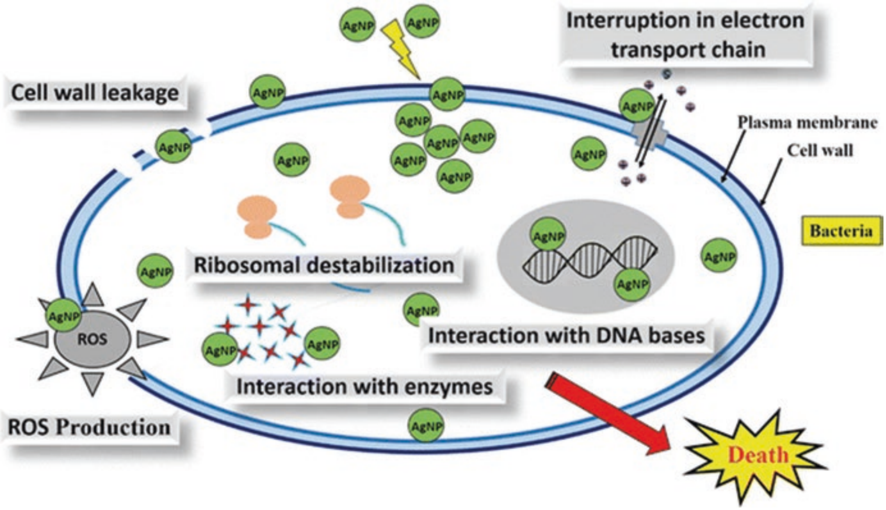

Fig. 7.3 Mechanisms of antibacterial activity for Ag NPs are diagrammatically represented. (With permission from Patil and Kim 2017)

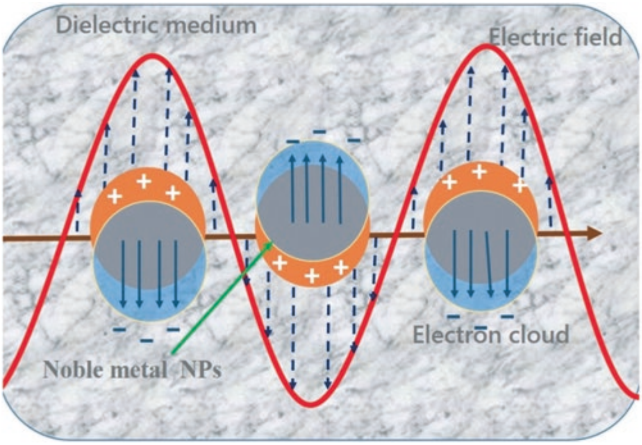

Fig. 7.4 Schematic representation of collective oscillations of surface electrons in response to incoming electromagnetic irradiation for noble metal NPs. (From Singh et al. 2017a)

recent reviews (Prasad et al. 2016; Singh et al. 2017a; Prakash et al. 2018). As discussed in the preceding section, TiO$_2$ is one of the most used antibacterial materials but has limited antibacterial application because of its wide bandgap. These noble metal NPs have been used extensively to enhance the visible light absorption properties of TiO$_2$ nanomaterials through SPR absorption.

Another advantage is that these plasmonic NPs form a Schottky barrier at the interfaces with TiO$_2$, acting as electron-trapping centers that inhibit the recombination of e^- and h^+ pairs, facilitating the oxidation and reduction reaction at the surface and producing ROS (Prakash et al. 2018). Therefore, the nanocomposite of Ag NPs with TiO_2 not only extends the application of TiO_2 into visible light but also enhances antibacterial activity from the synergic antibacterial activities of both (Prakash et al. 2018; Jai et al. 2016). The enhanced antibacterial activities of Ag–TiO_2 nanocomposites and the mechanism of their antibacterial action are discussed in the next section, with special emphasis on the engineering of their optical properties and antibacterial applications.

7.4 Ag–TiO_2 Nanocomposites: Mechanism and Antibacterial Applications

As already mentioned, TiO_2 is used widely for photocatalyst as well as antibacterial material, and extensive research has been carried out in different conditions (Li et al. 2017b). Also, its wide bandgap means TiO_2 is UV light activated and its photocatalytic antibacterial activities occur only under UV irradiation. The major drawback in this case is the fast recombination of photo-generated e^- and h^+ pairs, causing less efficient photocatalytic activity (Jai et al. 2016; Prakash et al. 2018). Therefore, visible light activation of TiO_2 is essential for proper utilization of its photocatalytic and hence antibacterial activities because sufficient visible light is present in the solar spectrum compared to UV light. Thus, not only are issues of fast recombination of charge carriers overcome but also additional improved physiochemical properties for multifunctional applications are available. Making nanocomposites with noble metals is one of the best strategies to extend the photocatalytic activity of TiO_2 to visible light (Fig. 7.5) (Wilke et al. 2018; Prakash et al. 2018).

The major advantage of combining noble metals with TiO_2 is the formation of the Schottky barrier at the interfaces with TiO_2, acting as an electron-trapping center that inhibits the recombination of e^- and h^+ pairs, facilitating the oxidation and reduction reaction at the surface and producing ROS under UV light irradiation. This combination also provides visible light-activated composite materials with enhanced optical properties that exhibit antibacterial activity not only under UV-visible light irradiation but also in dark conditions, that is, without light activation, from SPR absorption (Au/Ag) or antibacterial activity (Ag) (Fig. 7.5) (Wilke et al. 2018; Prakash et al. 2018).

In particular, composites of TiO_2 with Ag are most suitable for enhanced antibacterial activities of TiO_2-based nanomaterials. Ag not only enhances the optical properties of TiO_2 to improve its photocatalytic activities from its SPR properties but also synergistically enhances its antibacterial activities as an antibacterial agent. As Ag is also known as a good antibacterial material that works without any light activation, therefore its composites with TiO_2 (which antibacterial property is limited to UV activation) extend its implementation in the biomedical field broadly with or without use of light activation. In complementarity, in Ag–TiO_2 nanocomposites, TiO_2 serves as the base matrix for Ag NPs for better dispersion on the surface, which

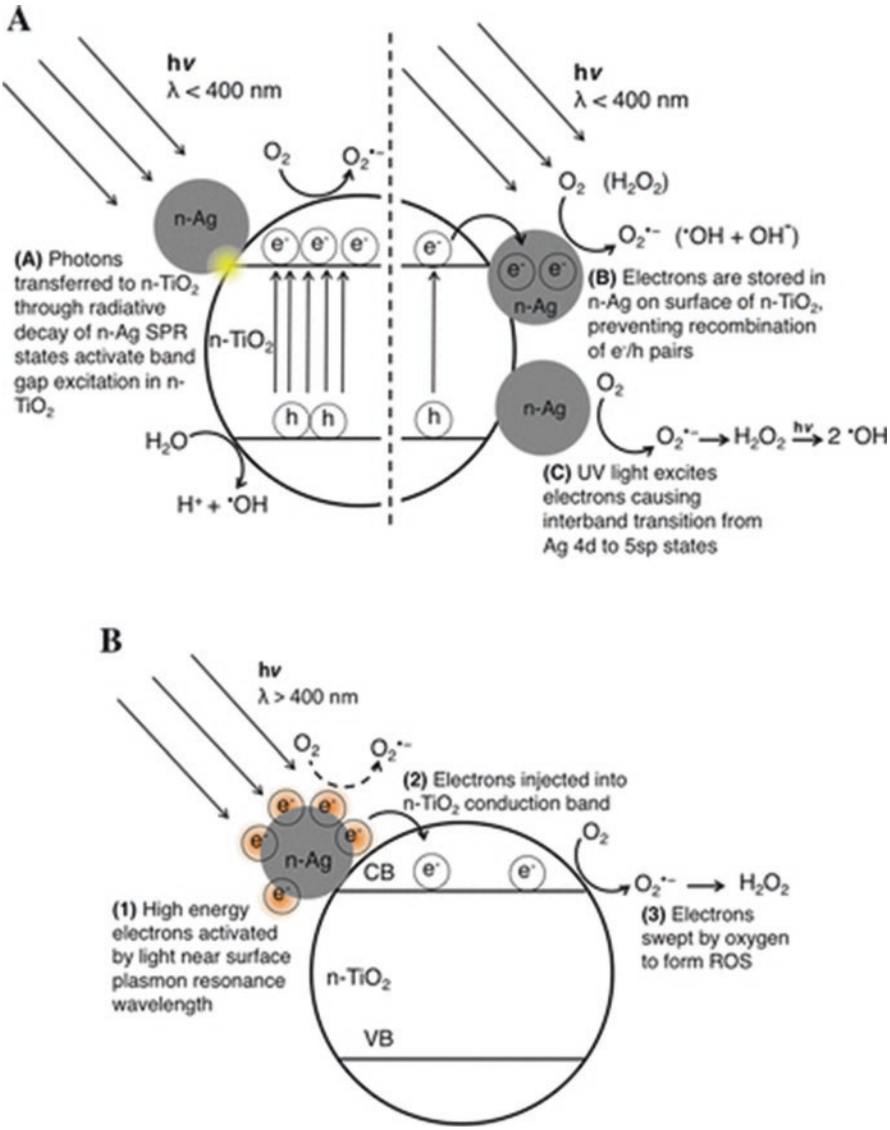

Fig. 7.5 Mechanism for photoactivity of n-Ag/n-TiO$_2$ and ROS production under ultraviolet radiation (**a**) and under visible light (**b**). (With permission from Wilke et al. 2018)

can be useful for optimizing the better SPR properties of Ag NPs (Prakash et al. 2018; Jai et al. 2016). In addition, it provides higher contact surface area for Ag NPs for uniform distribution on the surface. Several other factors such as structural modifications, the size and shape of Ag and TiO$_2$ nanomaterials, and their concentrations that provide efficient antibacterial materials with better antibacterial applications. For example, TiO$_2$ in a meso-porous structure is significant in antibacterial applications when used as nanocomposites with Ag NPs. The enhanced antibacterial response from these Ag–TiO_2 nanocomposites results from the high surface area in the meso-porous TiO_2 structure and slow release of Ag ions from the nanocomposites as compared to Ag NPs alone (Prakash et al. 2016; Akhavan 2009; Akhavan and Ghaderi 2009). Similarly, several studies have been based on different structures, shape, size, and concentration of Ag and TiO_2 nanostructures with enhanced antibacterial activities (Dror-Ehre et al. 2009; Pal et al. 2007; Machida et al. 2005). This section reviews the antibacterial applications of Ag–TiO_2-based nanocomposites in different conditions with emphasis on various factors that effectively enhance their antibacterial activities. The mechanisms of their antibacterial action under different conditions are also discussed.

Extensive research has been conducted on the antibacterial effect of Ag–TiO_2 nanocomposites under UV light irradiation. Matsunaga et al. (1985) studied for the first time the sterilization of bacteria using metal–TiO_2 nanocomposites activated with UV light that provided significant results with enhanced antibacterial effect as compared to UV-activated TiO_2 only. This experiment also provided better understanding of modifying TiO_2 through nanocomposite formation with various noble metal nanostructures (Ag/Au) to improve its antibacterial activity under UV irradiation, which encouraged researchers to work extensively in this direction (Keleher et al. 2002; Machida et al. 2005). Later, the nanocomposites of TiO_2 with these metal NPs were explored to study photocatalysis and antibacterial effects, not only in UV but also extended to work under visible light and dark conditions very effectively because of the synergic effect of light absorption and antibacterial effect of individual components, as discussed in Fig. 7.5 (Prakash et al. 2018; Wilke et al. 2018).

As discussed earlier and in the mechanism shown in Fig. 7.5, in case of Ag–TiO_2 nanocomposites under UV irradiation, the antibacterial effect is enhanced by the Schottky effect that promotes the charge separation along with antibacterial action of the Ag NPs. As a result, a number of e^- are present on the surface of TiO_2 that produce ROS or released Ag ions (especially in aqueous solutions) which accelerate the antibacterial action, providing higher antibacterial efficiency as compared to only Ag NPs or TiO_2 nanostructures (Keleher et al. 2002; Zhang et al. 2003). For example, Zhang et al. (2003) demonstrated that a nanocomposite formed by depositing Ag NPs on the surface of TiO_2 nanostructures showed better antibacterial activities under UV light as compared to TiO_2 only against *Micrococcus lylae*; charge separation followed by UV-generated ROS was mainly responsible for antibacterial activity as studied with transmission electron micrographs. Bahadur et al. (2016) presented similar observations against several bacterial cells that showed significant enhancement in antibacterial activities with increase in Ag concentration. It was suggested that Ag ions were released and attached to the cell membrane, affecting various functions of the cells and destroying the respiratory system, eventually inactivating the cell. However, the interaction also led to the formation of ROS, accelerating the killing of bacteria and thus enhancing the antibacterial effect (Fig. 7.6).

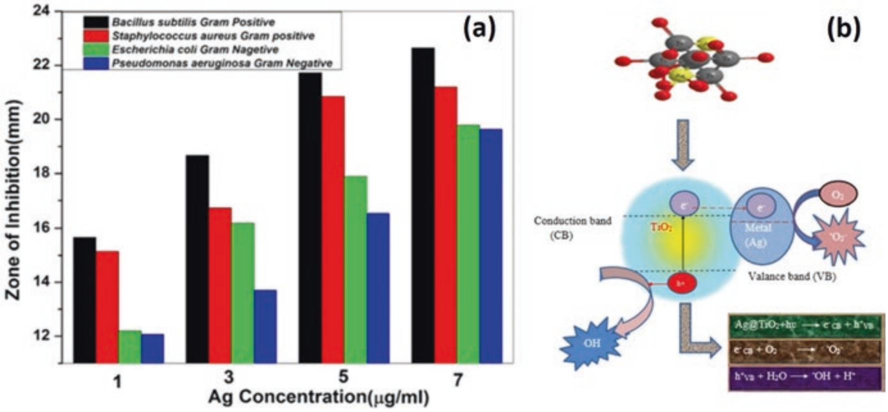

Fig. 7.6 (**a**) Bar graph for zone of inhibition versus Ag concentration. (**b**) Schematic diagram illustrating the mechanism of photo-excited electron and hole transfer among TiO$_2$ NPs and Ag NPs. (With permission from Bahadur et al. 2016)

In contrast, Page et al. (2007) did not observe any Ag ion release when antibacterial experiments were carried out in the dark using an Ag–TiO$_2$ nanocomposite coating. The activity was explained on the basis of charge separation and ROS formation leading to the death of the cells. It was found that antibacterial behavior was different for Gram-positive and Gram-negative bacteria because of the difference in cell wall structures. Similarly, Ubonchonlakate et al. (2012) showed that under UV irradiation, Ag–TiO$_2$ nanocomposite films exhibited better (100% antibacterial efficiency) effect as compared to TiO$_2$ whereas TiO$_2$ in pure and porous structures showed only 57% and 93% efficiency, respectively. Interestingly, it was found that 100% killing disinfection could be achieved in less UV irradiation time as compared to TiO$_2$ irradiation time as Ag attributed to the higher charge separation. Several studies on Ag–TiO$_2$-based nanocomposites for their antibacterial application have been reported under UV irradiation that indicated enhanced efficiency from the presence of Ag NPs. However, several other factors generally affect antibacterial activities such as preparation methods, kind of bacteria, structural composition, thickness of the nanocomposites in case of thin films, different phases of the TiO$_2$, and experimental parameters such as temperature, darkness, or electromagnetic irradiation (Machida et al. 2005; Page et al. 2007; Kubacka et al. 2008). It has been found that in any case, however, the Ag–TiO$_2$ nanocomposites exhibit better antibacterial response as compared to only Ag or TiO$_2$ nanomaterials (Akhavan 2009; Deshmukh et al. 2018; Jai et al. 2016; Jalali et al. 2016; Kubacka et al. 2008; Prakash et al. 2018; Chiang et al. 2014). For example, varying Ag composition in an Ag/Au–TiO$_2$ nanocomposite where alloy Ag/Au NPs were formed on the TiO$_2$ surface showed better antibacterial killing effect under the influence of UV irradiation as compared to visible light (Chiang et al. 2014). Page et al. (2007) studied the antibacterial effect of TiO$_2$ and Ag–TiO$_2$ coating on glass slides and found this very effective under the influence of UV irradiation (Fig. 7.7). These coatings were very effective against Gram-positive bacteria as compared to Gram-negative bacteria

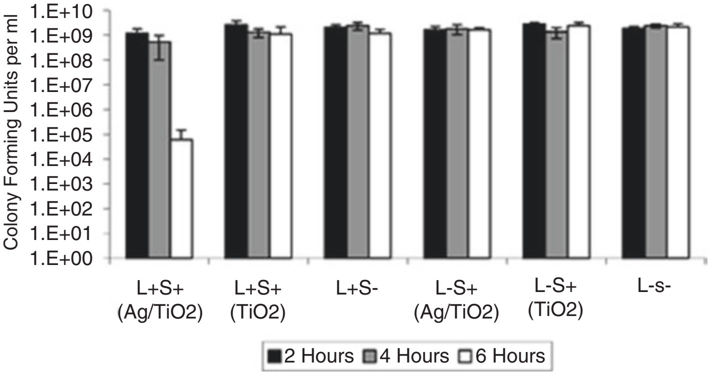

Fig. 7.7 Bacterial kills for the two-coat Ag–TiO_2 sol–gel-prepared coating against *Staphylococcus aureus* after 2, 4, and 6 h illumination with 365 nm radiation. The viable counts are expressed as colony-forming units ml^{-1}. L+S+ refers to the exposure of an active coating (identity in brackets) to UV light; L+S− refers to the exposure of an uncoated slide to UV light; L−S+ refers to an active coating (identity in brackets) kept in the dark; and L−S− refers to an uncoated slide kept in the dark. (With permission from Page et al. 2007)

where the charge separation mechanism and structural composition of the cell wall had major effects on the observed differences in antibacterial activities.

The preparation methods also showed differences in antibacterial efficiency under UV irradiation. For example, a comparable antibacterial study was performed by synthesizing an Ag–TiO_2 nanocomposite using impregnation and photodeposition methods against *E. coli* bacteria. Although nanocomposite Ag–TiO_2 materials prepared by both techniques exhibited good antibacterial effect, nanocomposites prepared by the photo-deposition method showed superior antibacterial effects (Kubacka et al. 2008). Interestingly, these Ag–TiO_2 nanocomposite materials are also very effective in dark conditions against various microbes (Binyu et al. 2011; Li et al. 2011). In dark conditions, the noble metal NPs deposited on TiO_2 surfaces can absorb the electrons from the bacteria by their Plasmon, and these electron transfers from the bacteria to interrupt their respiratory system leading to cell death (Li et al. 2011; Prakash et al. 2018). Binyu et al. (2011) studied the antibacterial effect of Ag–TiO_2 nanocomposites in darkness and compared the antibacterial results under the effect of UV light. In both experimental conditions, these Ag–TiO_2 nanocomposites showed excellent antibacterial response against *E. coli*.

So far, we have discussed the antibacterial effect of Ag–TiO_2 nanocomposites under UV light irradiation and found that the limitation of UV light activity of TiO_2 nanomaterials decreases the antibacterial activity efficiency. However, the presence of Ag NPs and formation of the Schottky barrier at the interface leads to suppression of recombination of charge carriers, providing more electrons and holes to take part in oxidation and reduction reactions, generating ROS and further inactivation of microbes. In addition, we have studied the antibacterial effect in dark conditions. Interestingly, in visible light, antibacterial activity is found to be enhanced as compared to that of UV light irradiation because of the localized surface plasmon resonance (LSPR) properties of Ag NPs (Nigussie et al. 2018; Prakash et al. 2018). The presence of Ag NPs in Ag–TiO_2 nanocomposites increases absorption of visible light, which excites the electrons in LSPR. These excited electrons are transferred to the CB of TiO_2 for further formation of ROS through oxidation/reduction reactions that take part in either photocatalytic or antibacterial activities (Fig. 7.5) (Wilke et al. 2018).

Wilke et al. (2018) recently demonstrated that because of the photoactive nature of both Ag and TiO_2 under visible light irradiation, they showed better interaction for producing ROS that exerted toxic stress on bacterial cells, leading to damage of cell membrane and death. They also studied the similar behavior of Ag–TiO_2 nanocomposites in dark conditions (Wilke et al. 2016). Jiang et al. (2017) reported that these materials caused a slight lesion in the cell membrane leading to inhibiting cell growth. Similarly, from the formation of enhanced ROS under visible light, Ali et al. (2018) showed enhanced photocatalytic degradation as well as photocatalytic antibacterial activity against various microbes. Mai et al. (2010) studied the antibacterial behavior of an Ag–TiO_2 nanocomposite prepared by the sol–gel method in different conditions such as dark, heating, and visible light irradiation. There was not much effect of annealing on antibacterial activity, whereas in the dark as well as under visible light, these nanocomposites show better antibacterial response because of the Ag^+ release and plasmonic effect, respectively. A similar mechanism was proposed for Ag–TiO_2 film deposited on Ag NPs over the TiO_2 substrate by Akhavan et al. Akhavan (2009) demonstrated excellent antibacterial activities under visible, solar, as well as dark conditions. Several studies using systematic studies have proposed a similar mechanism for Ag-TiO_2 based nanocomposites (Bahadur et al. 2016; Zhao et al. 2011; Roguska et al. 2012).

Ag–TiO_2-based nanocomposites have also been used for studying toxicity effects on human cells (Korshed et al. 2017) and inhibition of biofouling on limestone (Becerra et al. 2018). Korshed et al. (2017) reported antibacterial action and toxicity effect of a novel Ag–TiO_2 nanocomposite prepared from picosecond laser against several microbes (*E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) and human cells (A549, HePG2, HEK293, hCAECs, and HDFc), respectively, under visible light treatment. The visible light activation caused an enhancement in the level of ROS as compared to TiO_2 that had a major role in lipid peroxidation, depletion of cell membranes, and leaking of compounds, followed by death of the microbes. Similarly, decreased cell proliferation was recorded in human cells when treated with these Ag–TiO_2 nanocomposites. On the other hand, Cao et al. (2017) demonstrated that Ag–TiO_2 nanocomposites produced by the strong electrostatic adsorption (SEA) technique resulted in fine and well-distributed Ag NPs on the TiO_2 surface and exhibited excellent antibacterial response. They concluded that fine dispersion and small size of Ag NPs had a major effect on enhanced antibacterial action (Fig. 7.8).

Smaller Ag NPs are effective for releasing Ag^+ and can penetrate the cell membrane, followed by entering the cell and damaging cell functions. It was shown that SEA-based Ag NPs were releasing Ag^+ in a controlled manner, providing long-term antibacterial functionality. As shown in Fig. 7.8, the SEA-generated Ag NPs distributed

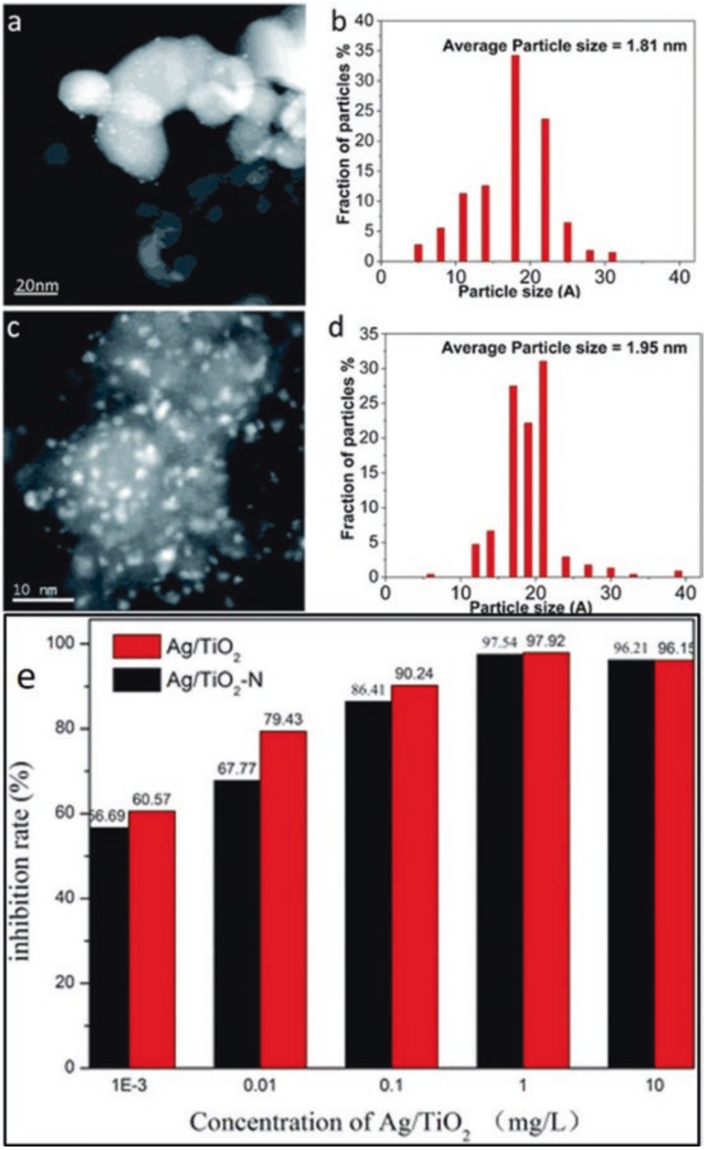

Fig. 7.8 Scanning transmission electron microscopy (STEM) image of Ag/TiO_2–N (**a**), Ag/TiO_2 (**c**), and particle size distribution (**b**, **d**). (**e**) Antibacterial rate of different concentrations of Ag/TiO_2. (From Cao et al. 2017)

over TiO_2 exhibited antibacterial activities through Ag^+ release and ROS formation. leading to inactivation of bacterial cells.

Cai et al. (2018) recently proposed a memory catalyst (MC) material based on Ag–TiO_2 nanocomposites that showed unique catalytic performance during the dark

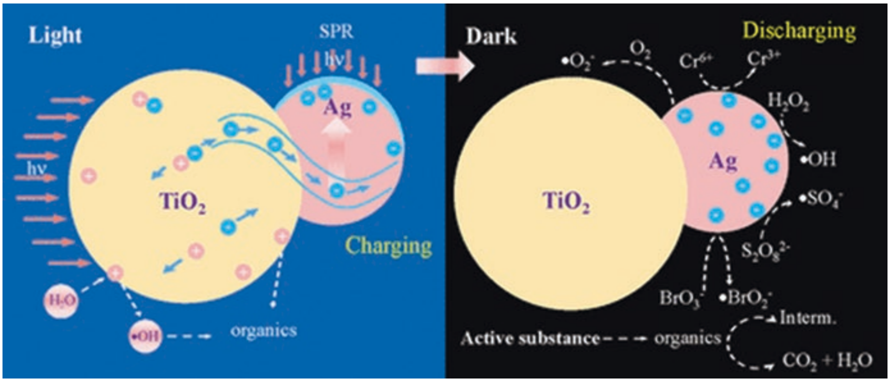

Fig. 7.9 Memory catalyst (MC) reaction mechanism. (With permission from Cai et al. 2018)

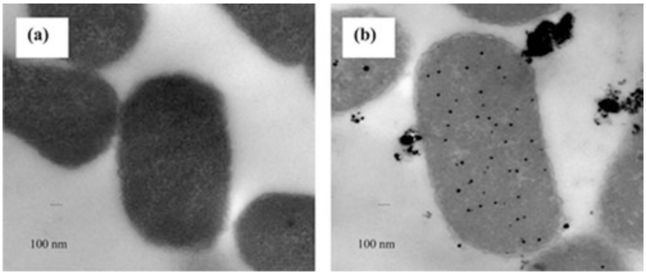

Fig. 7.10 TEM images of *E. coli* cells before (**a**) and after (**b**) treatment with 10% Ag/(C, S)–TiO_2 nanoparticles. (With permission from Hamal et al. 2010)

as a result of electron-storing ability through the electron-trapping effect of TiO_2 nanomaterials. It was suggested that these material could be the best plasmonic photocatalytic materials for antibacterial applications even without any influence of light irradiation (Fig. 7.9).

In other studies, it has been shown that Ag–TiO_2 nanocomposites show better antibacterial performance when forming multi-element composites by incorporating or doping other functional elements. For example, Hamal et al. (2010) demonstrated that Ag–TiO_2 nanocomposites when doped with functional elements such as C or S exhibited better multifunctional photocatalytic activities. They studied photocatalytic degradation as well as photocatalytic antibacterial activities of doped Ag–TiO_2 nanocomposites and found enhanced antibacterial activity against *E. coli* under visible light as well as dark conditions, attributed to the enhanced Ag^+ ion release caused by doping (Fig. 7.10).

Similarly, Jiang et al. (2016) suggested that a composite of Ag–TiO_2 with graphene oxide would be excellent for its antibacterial action attributed to the better release of Ag^+ (Fig. 7.11).

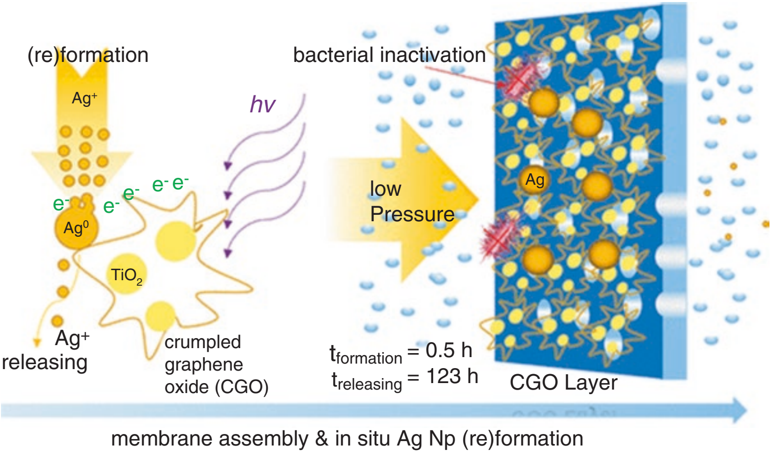

Fig. 7.11 Ag NP–TiO_2 on graphene oxide for antibacterial applications. (From Jiang et al. 2016)

Similarly, when these Ag–TiO_2 nanocomposites are embedded in some soft matrix such as polymer, they are more attractive from long-term stability and technological aspects. For example, Noori Hashemabad et al. (2017) reported that when TiO_2 nanoparticles were incorporated in polyethylene films, the antibacterial property was enhanced and the composites could be used as for food packaging to avoid such contaminations. Much study has been conducted using TiO_2 and polymer nanocomposites for enhanced antibacterial activity in different technological applications (Varnagiris et al. 2017; Kong et al. 2010; Santhosh and Natarajan 2015; Kubacka et al. 2009b). Embedding in a polymer matrix provided not only protection to the nanomaterials but also provided better stability from the open environment, specially for Ag NPs, which can be easily oxidized (Singh et al. 2013; Prakash et al. 2018). The nanocomposites of Ag–TiO_2 with polymer showed even better antibacterial activity as compared to Ag–TiO_2 nanoparticles only (Kubacka et al. 2009a; Tallósy et al. 2014; Wang et al. 2016). Tallósy et al. (2014) investigated the antibacterial response of TiO_2, Ag–TiO_2, and Ag–ZnO nanoparticles embedded in polymer films. They observed that in similar experimental conditions, Ag–TiO_2/polymer nanocomposite films showed better antibacterial activities as compared to either TiO_2/polymer or Ag-ZnO/polymer films in the dark as well as visible light irradiation (Fig. 7.12).

Similarly, Wang et al. (2016) demonstrated that Ag-poly(dopamine)–TiO_2 nanotube composites exhibited very stable and long-term antibacterial activity against microbes as a result of slow Ag^+ release from the polymer layer. Similarly, polyurethane acrylate-Ag/TiO_2 nanorod-based nanocomposites exhibited strong antibacterial photodegradation of *E. coli* when irradiated with UV light through ROS generation (Li et al. 2017a). In another report, the plasmonic effect of Ag–TiO_2 NPs embedded in polymer films decreased the amount of plasmonic photocatalyst and thus exhibited great enhancement in the antibacterial killing efficiency of the composites (Kubacka et al. 2009a; Wu et al. 2010)**.** These polymer-based plasmonic

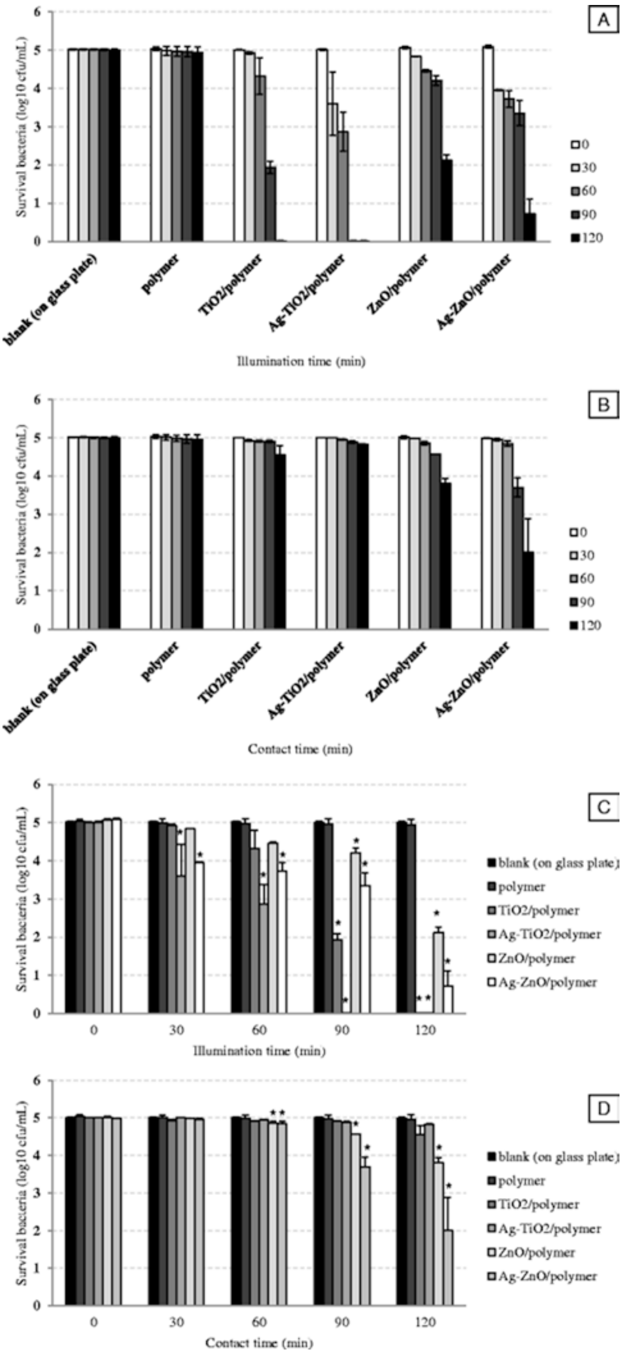

Fig. 7.12 Antibacterial effect of Ag–ZnO/polymer and Ag–TiO$_2$/polymer nanohybrid films against methicillin-resistant *Staphylococcus aureus* (MRSA) under LED light illumination (**a**) and in the dark (**b**), and with statistical analysis [under LED light illumination (**c**) and in the dark (**d**)]. (With permission from Tallósy et al. 2014)

photocatalysts have been efficiently used in packaging and biomedical applications (Kubacka et al. 2009a, b).

Similarly, Ag–TiO_2-polyethylene-based nanocomposites were used to inactivate pathogens by examining disinfection against various microbes such as *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger* (Barani et al. 2018). Cross-linked polymer-based hydrogel nanocomposites embedded in Ag NP–TiO_2 nanosheets exhibited excellent antibacterial activity with strong photocatalytic degradation of dye molecules (Sarkar et al. 2017). Polyvinylidene fluoride (PVDF)-Ag–TiO_2-based nanocomposite membranes used as bifunctional materials exhibit excellent photocatalytic and antibacterial properties. The excellent bifunctionality was attributed to the introduced hydrophilicity of the Ag–TiO_2 nanocomposite materials (Chen et al. 2017). PVDF membranes produced by blending Ag–TiO_2–APTES nanocomposites were also shown to be effective bifunctional and self-cleaning materials for antibacterial as well as photodegradation applications (Peng et al. 2018).

7.5 Outlook and Summary

Ag–TiO_2 nanocomposite nanomaterials are important functional materials that are being used for several applications, including bacterial disinfection. These nanocomposites, in particular, are very useful in antibacterial activities by means of the synergic effect of Ag NPs and TiO_2. These nanomaterials have gained much attention from researchers because their synthesis process is easy and their physicochemical properties are excellent as well as tunable. Another important aspect about these nanocomposite materials is their antibacterial application in various conditions such as in dark and under UV and visible/solar light irradiation, attributed to the presence of Ag and TiO_2, respectively. In this chapter, recent progress on novel Ag–TiO_2 nanomaterials in the field of antibacterial applications is discussed with emphasis on the advances of mechanisms of antibacterial action in recent years as reported in the literature.

The chapter is divided in four sections. In Sect. 7.1, a general introduction on bacterial infection, need of antibacterial materials, and importance of Ag-TiO_2 nanomaterials are discussed. Section 7.2 describes the basics and antibacterial mechanism of TiO_2 materials; Sect. 7.3 discusses in brief the Ag NPs and their role in antibacterial applications. Section 7.4 provides a detailed overview of recent developments in antibacterial applications of Ag-TiO_2 nanocomposites with emphasis on antibacterial mechanisms. We hope this chapter will provide useful information about progress in the antibacterial research field.

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Chapter 8
Ecotoxic Effect of Photocatalytic Active Nanoparticles on Human Health and the Environment

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8.1 Introduction

Nanotechnology is a rapidly expanding industry that covers many areas of science and technological utilization. This technology has already yielded a variety of commercial products such as cosmetics, medical science equipment, self-cleaning windows, paints, and stain-resistant clothing. For personal care products, some nanoparticles (NPs) such as zinc oxide (ZnO) and titanium dioxide (TiO_2) are applied in toothpaste, sunscreens, and beauty products (Serpone et al. 2007). Similarly, Ag NPs are used as antimicrobial additives in food packaging, detergents, and textiles such as socks and underclothes (Maynard and Michelson 2006). The integration of nanomaterials in the life of consumers has caused increased concern regarding hazards to the environment and to human health. Accidental exposure to the nanoparticles may arise at three stages: (1) during production of NPs, (2) via release from the solar/coatings/batteries or during separation of the material, and (3) after recycling/disposal.

Discussions are suggested by warnings and complaints from researchers and toxicologists that arise from the challenges correlated with the toxicity evaluation of nanomaterials. When the whole environment is concerned, the issue is more intricate. Although there was formerly a considerable amount of toxicological information concerning available NPs (obtained at different biological levels), ecotoxicological data on nanoparticles are just emerging (Kahru and Dubourguier 2010).

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However, there is a considerable amount of experience and data on environmental risk evaluation of chemicals. The main properties of NPs, such as high specific surface area, large reactive sites on the surface that cause an extensive fraction of atoms to be located on the external rather than in the internal of NPs, and also their mobility, can cause unexpected health or environmental risks (Maynard et al. 2006; Wiesner et al. 2006; Prasad et al. 2016). Accordingly, organisms, and particularly those that interact with their environment such as aquatic plants and algae, are expected to be affected as a consequence of their exposure to nanoparticles.

In this chapter, the environmental treatment and ecotoxicity of nanoparticles to organisms such as algae and aquatic plants are investigated. First, the behavior of NPs in aquatic systems as the basic living place for algae and plants are discussed, and then the toxicity of NPs is examined.

8.2 Photocatalytic Nanomaterial

Photocatalytic nanoparticles have an important function to apply an impressive photocatalytic reaction, and therefore it is important that we select them carefully to carry out both acceptable energy of light and proper electronic structure for its photoactivation (Ohtani 2013). Photocatalysis semiconductor NPs accomplish the requirements of photocatalysts, and the bandgaps are used to explain their electronic structure, which is characterized by the valence band (VB) and the conduction band (CB), which is located on top of the valence band and separated from it by an energy gap, named the bandgap (Eg) (Hernández-Ramírez and Medina-Ramírez 2015). Photocatalytic process are based on electronic excitation of the catalyst (semiconductor) by UV light irradiation. By this excitation of semiconductor with energy from photons, and equal or higher energy bandgap of semiconductor, the electron is promoted from VB of semiconductor to CB, thus leaving a positive hole (h+) on the VB. The promoted electrons and holes migrate to the surface of the photocatalyst and the oxidation-reduction process, respectively, occurs (Fig. 8.1) (Pichat 2007).

Semiconductors are also classified as binary and ternary compounds; however, the appropriate semiconductors for photocatalysts must achieve the following: have an appropriate bandgap energy (1–4 eV), high stability, and an appropriate band edge place that matches the intended use radiation absorption and nontoxicity (Hernández-Ramírez and Medina-Ramírez 2015). The usual classes of photocatalytic nanoparticles are illustrated in Fig. 8.2.

Most photocatalytic NPs and composites are metal oxides. Such binary oxides as titanium dioxide (TiO_2), tungsten oxide (WO_3), zinc oxide (ZnO), and iron oxide (Fe_2O_3) are the most studied semiconductors in photocatalytic experiments. TiO_2 is mostly used in different technical applications because of its hydrophilicity, stability, high reactivity, and low cost (Schneider et al. 2014). The best known polymorphs of TiO_2 NPs are rutile, anatase, and brookite with bandgap energies of 3.02, 3.20, and 2.96, respectively (Gupta and Tripathi 2011). The anatase phase displays

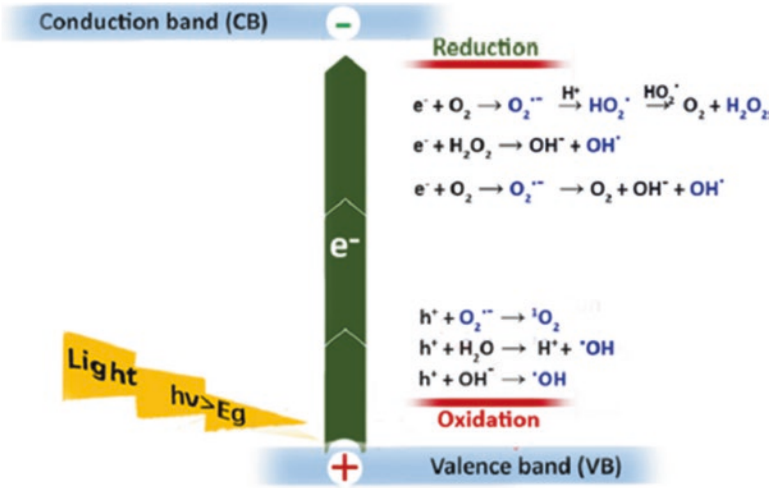

Fig. 8.1 The mechanism of reactive oxygen species (ROS) generation

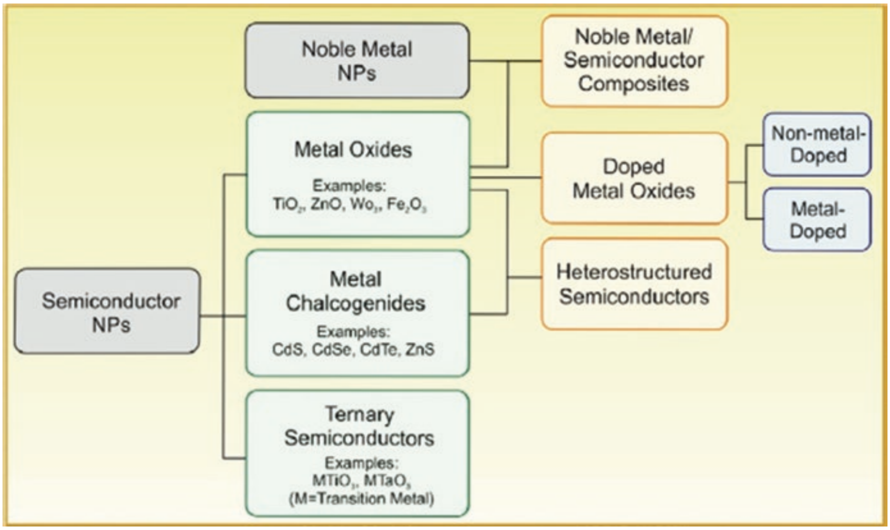

Fig. 8.2 Classes of photocatalytic nanoparticles (NPs) and their composites. (From Friehs et al. 2016)

the highest photocatalytic performance and thus it is applied in photocatalytic processes. The rutile phase is applied as a thickener and pigment in cosmetic products and other uses for its UV light-absorbing properties (Mueller and Nowack 2008). Hence, brookite and anatase are used in dye-sensitized solar cells (Jiang et al. 2002). Such characteristics have led to the use of TiO_2 NPs for different applications, such as light-emitting diodes, self-cleaning surface coatings, disinfectant sprays, solar cells, water treatment agents, and topical sunscreens (EPA 2009). However, TiO_2 NPs have many different sources. Degussa P25 is known as the standard nanoparticle in the field of photocatalysts as its well-defined nature consists of rutile and anatase phases in a ratio of 1:3 (Friehs et al. 2016). TiO_2 NPs are applied in sunscreen cosmetics as an UV absorbent. To decrease its photo-reactivity, nano silica and other compounds can be added to TiO_2 NPs as a surface coating; hence, TiO_2 NPs can protect human skin and plastics from UV irradiation (EPA 2009). ZnO nanoparticles provide good catalytic and photochemical properties; hence, it is considered as the most applicable photocatalytic semiconductor after TiO_2 (Hatamie et al. 2015). Because of its high quantum efficiency, ZnO is usually preferred in comparison to TiO_2 to degrade and decompose organic contaminants (Farbod and Jafarpoor 2012). The main disadvantages of ZnO NPs are the possibility of the photo-corrosion phenomenon at low pH values (Di Paola et al. 2012). The bandgap energy of the ZnO semiconductor is 3.37 eV, with a wide excitation binding energy (60 eV), and presents near-UV light irradiation, piezoelectricity, that is more attractive in electronic sensor and transparent conductivity. ZnO NPs are extensively used in products such as foods (source of Zn nutrient), ceramics, plastics, cement, glass, rubber, fire retardants, paints, lubricants, pigments, and batteries. Moreover, ZnO nanoparticles have been used in personal care products such as cosmetics and sunscreens because of their superior UV absorption and reflective characteristics. The production of nanoparticles for only sunscreen cosmetic products was estimated to be about 1000 tons between 2003 and 2004, consisting of ZnO and TiO_2 NPs (Borm et al. 2006).

8.3 Nanoparticles as Potential Aquatic Contaminants

Industrial wastes and products tend to be released to the aquatic environment, such as rivers, estuaries, drainage ditches, lakes, and coastal waters, despite protections. Accordingly, as the nanoproducts industries start large-scale production, it is certain that nanoscale products will be discharged into surface waters. Thus, a crucial issue is that we have beneficial risk assessment procedures in place in the fastest possible time to handle potential hazards.

Current studies indicate that the concentration of ZnO NPs in the UK environment range from less than 100 μg/l in water to a few milligrams per kilogram (mg/kg) in soil (Boxall et al. 2007); as well, a further study by Gottschalk confirmed ZnO NPs concentration as about 10 ng/l in natural waterways and 430 ng/l in wastewater in Europe (Gottschalk et al. 2009) (Table 8.1).

Moreover, studies observe that the metallic Ti concentrations found in the surface water are about 600 mg/l (Kägi et al. 2008). Kiser et al. (2009) estimated the levels of Ti nanomaterial release from wastewater plants: raw sewage contains 100–3000 mg/l of Ti. Ti concentrations in wastewater effluents ranged from <5 to 15 mg/l. Two studies modelled the TiO_2 NPs discharged into the environment (Gottschalk et al. 2009; Mueller and Nowack 2008); the estimated environmental

Table 8.1 Modeled concentrations of TiO_2 and ZnO nanoparticles (NPs) released into environmental compartments in different countries, shown as mode (most frequent value) and as range of the lower and upper quantiles ($Q_{0.15}$ and $Q_{0.85}$)

Environmental compartment	Predicted environmental concentration, mode ($Q_{0.15}$, $Q_{0.85}$)[a] (mg/l, mg/m^3, or mg/kg)		
	Switzerland	Europe	U.S.
TiO_2 NP			
Surface water	0.021 (0.016, 0.085)	0.015 (0.012, 0.057)	0.002 (0.002, 0.010)
STP effluent	4.28 (3.50, 16.3)	3.47 (2.50, 10.8)	1.75 (1.37, 6.70)
STP sludge	211 (172, 802)	136 (100, 433)	137 (107, 523)
Sediment	499 (426, 2382)	358 (273, 1409)	53 (44, 251)
Soil	0.28 (0.21, 1.04)	1.28 (1.01, 4.45)	0.53 (0.43, 2.13)
Sludge-treated soil		89.2 (70.6, 310)	42.0 (34.5, 170)
Air	0.001 (0.0007, 0.003)	<0.0005	<0.0005
ZnO NP			
Surface water	0.013 (0.011, 0.058)	0.010 (0.008, 0.055)	0.001 (0.001, 0.003)
STP effluent	0.441 (0.343, 1.32)	0.432 (0.340, 1.42)	0.3 (0.22, 0.74)
STP sludge	21.4 (16.8, 64.7)	17.1 (13.6, 57.0)	23.2 (17.4, 57.7)
Sediment	3.33 (3.30, 56.0)	2.90 (2.65, 51.7)	0.51 (0.49, 8.36)
Soil	0.032 (0.026, 0.127)	0.093 (0.085, 0.661)	0.050 (0.041, 0.274)
Sludge-treated soil		3.25 (2.98, 23.1)	1.99 (1.62, 10.9)
Air	<0.0005	<0.0005	<0.0005

From Gottschalk et al. (2009)

[a] For surface water, air, and sewage treatment plant (STP) effluents, the results obtained are current 2008 nanoparticles concentrations; for soil, sludge-treated soil, and sediments, the annual increase of engineered nanoparticle (ENM) concentration

concentrations are exhibited in Table 8.1. The estimated concentrations of TiO_2 NPs in consumer products were 1030 mg/kg for soil and 24.5 mg/l for water (Boxall et al. 2007). It is predicted that once nanoparticles are in the water, they will most likely agglomerate and partition to suspended particulate matter and sediment (Boxall et al. 2007). Agglomerated particles are usually less mobile and may interact with sediment-dwelling organisms and filter feeders (Farré et al. 2009; Menard et al. 2011). Humic acids have been noted to have considerable influence on the agglomeration of TiO_2 NPs (Pettibone et al. 2008). Many studies have already described the effects of ZnO and TiO_2 NPs on biological systems. TiO_2 and ZnO NPs have been shown to generate reactive oxygen species (ROS) in the absence and presence of UV radiation (Armelao et al. 2007; Reeves et al. 2008). The accurate mechanisms of toxicity of ZnO, TiO_2, and other metal NPs are generally unknown (Griffitt et al. 2008), but recent studies have presented that the toxicity of NPs is essentially influenced by properties such as shape and morphology, particle size, and surface properties (Crane et al. 2008). There is emerging knowledge on the ecotoxicity of ZnO and TiO_2 NPs, with many studies related to aquatic organisms, algae, fish, and freshwater invertebrates. Recently, invertebrates, algae, and fish species were tested: freshwater invertebrates are the most studied group for which the most data exists, and after that algae and then freshwater fish. Similar data among these groups of organisms were also obtained for other NPs (Cattaneo et al. 2009). As there is a lack of available knowledge on the toxic effects of TiO_2 and ZnO NPs on sedimentary, terrestrial, and aquatic plants, research in the field is needed.

8.4 Bioavailability of Nanoparticles in Different Environments

Ecotoxicological tests are tools applied within ecological hazard assessment frameworks to understand the aquatic toxicology and intrinsic dangers of chemical compounds that leach into the environment. The term ecotoxicity was mainly developed for water-soluble chemical substances and aquatic test organisms. However, in spite of the apparent path of exposure and the mechanisms of ecotoxicity, bioavailability exists as a key factor for the hazard assessment of synthetic nanoparticles. Bioavailability is a dynamic intention that evaluates the physical, chemical, and biological processes of exposure. Bioavailability combines concepts of ecotoxicology and environmental chemistry, incorporating concentration of contaminant and an organism's treatment in the given environment. Bioavailability of nanoparticles depends on the physicochemical characteristics of the particles (solubility and aggregation) and behavior of nanoparticle–organism in the particular environment. Hence, environmental hazard assessment of NPs needs characterization of NPs in entire processes of exposure.

8.5 Ecotoxicity of Nanoparticles

8.5.1 Entry of Nanoparticles into Algae, Aquatic Plants, and Fungi

Apart from the animals, most cells of algae, plants, and fungi contain cell walls that form a primary site which acts as a barrier for the entry of NPs into their cells. The majority of cell wall components are proteins and carbohydrates that are connected to form a hard complex network (Knox 1995). The functional groups, such as phosphate, carboxylate, amine, imidazole, hydroxyls, and sulfhydryl are included in these biomolecules (Vinopal et al. 2007). Cell walls in algae and plants mostly consist of cellulose. Moreover, algae have polysaccharides and glycoproteins in their cell walls. The semipermeable properties of cell walls can permit small molecules to cross cell walls, whereas for larger molecules they are limited. The pore diameter of cell walls ranges from 5 to 20 nm (Fleischer et al. 1999; Zemke-White et al. 2000).Thus, only NPs with a diameter smaller than the pore size are expected to cross through the cell wall. Moreover, the interactions of the cells with NPs might influence the pores of the cell wall and form a new pore size that might be larger than usual and hence allow increased entry of NPs through the cell wall and after that to conjoin the plasma membrane. Currently, the entry routes of nanoparticles through this lipid membrane have been debated (Moore 2006). In the endocytic process, the plasma membrane generates a hole structure that surrounds the NP and moves it into the cell. The NPs might pass the cell membranes using blended transport carrier proteins. Upon the entry of the NP into the cell, they may attach to organelles such as the endoplasmic reticulum, the endolysosomal system, and Golgi apparatus and then hamper those metabolic processes. Moreover, at high concentrations of NPs, the nucleus of macrophage cells may display degeneration, rarefaction, and enlargement (Jia et al. 2005).

8.5.2 Effects of Nanoparticles on Algae and Aquatic Plants

Although there is little information on the effects of NPs on algae and plants, both direct and indirect effects have been found. Direct ecotoxic effects of nanoparticles on organisms are mainly obtained by their particle size, surface reactivity, and chemical composition. Some ecotoxicological effects of TiO_2 NPs on algae have been obtained in recent years (Kahru and Dubourguier 2010; Klaine et al. 2008). Ecotoxicity tests for algae haves been evaluated with three species: *Desmodesmus subspicatus*, *Pseudokirchneriella subcapitata*, and *Chlamydomonas reinhardtii*. Growth inhibition was usually measured after 72 h (Aruoja et al. 2009; Blaise et al. 2008; Hund-Rinke and Simon 2006) or 96 h (Griffitt et al. 2008), and different 72-h EC_{50} values were studied for TiO_2 NPs. For example, in *P. subcapitata*, values ranged from 5.83 mg/l of Ti (Aruoja et al. 2009) to 241 mg/l of TiO_2 (Hartmann et al. 2010). The 72-h EC_{50} of TiO_2 assessed for *D. subspicatus* was 32 mg/l (mainly anatase, particle size of 25 nm), but when another TiO_2 form (100% anatase, particle size of 100 nm) was measured, there was no effect at ranges less than 50 mg/l of TiO_2 (Hund-Rinke and Simon 2006). Acutely variable LC_{50} and 72-h EC_{50} values were measured for TiO_2 NPs tested with *P. subcapitata* (Table 8.2). No clear correlations between the particle size and effects on algae *P. subcapitata* could be recognized. For example, nanoparticles described at levels below 100 nm in diameter were not totally toxic to these algae (Blaise et al. 2008), whereas sonicated nanoparticles with sizes between 25 and 70 nm in diameter were much more toxic (Aruoja et al. 2009). On the other hand, evidence indicated that the toxicity of two sizes (<10 nm and 30 nm) of TiO_2 NPs did not cause much difference, and thus no clear dependence between the particle size and effects was observed (Hartmann et al. 2010).

A relationship between the nanoparticle-specific surface area and effect concentrations is shown in Fig. 8.3a. With increasing specific surface area, the toxicity of TiO_2 NPs to the alga *P. subcapitata* decreases. For example, the particles with a specific surface area of 288 m^2/g are less toxic to *P. subcapitata* than the particles with a specific surface area of 5.8 m^2/g. Moreover, as shown in Fig. 8.4 and Table 8.3,

Table 8.2 Physicochemical properties of TiO_2 NPs and toxicity values for the alga *Pseudokirchneriella subcapitata*

Species	Particle size (nm)[a]	Crystal phase	BET (m^2/g)[b]	DLS (nm)[c]	Toxicity value (mg/l)
P. subcapitata	/	w99% TiO_2 core with 1% Al surface coating	5.8	380	$EC_{50} = 16$ (12–22) (conc. not measured) $EC_{50} = 61$ (52–72) (nominal conc.)
	/	79% rutile/21% anatase; 90 wt% TiO_2, 7% alumina, 1% amorphous silica	38.5	140	$EC_{50} = 21$ (16–26) (conc. not measured) $EC_{50} = 87$ (83–91) (nominal conc.)
	<10	67.2% anatase/32.8% amorphous	288	1261	$EC_{50} = 241$ (95.6–609)
	25–70	/	/	/	$EC_{50} = 5.83$ (3.75–7.58)
	30	72.6% anatase/18.4% rutile/9% amorphous	47	416	$EC_{50} = 71.1$ (59.4–85.1)
	<100	99.9% TiO_2	/	/	$IC_{25} > 100$

From Menard et al. (2011)

/ no data available, *EC_{50}* median effective concentration, *IC_{25}* 25% inhibition concentration, ( ) = 95% confidence intervals

[a] Particle size reported by the manufacturer

[b] Specific surface area calculated with Brunauer, Emmett, and Teller method (BET)

[c] Median values for particle size in media determined with dynamic light scattering (DLS)

the larger surface area per mass, compared with greater particle sizes of the same component, presents NPs as more reactive biologically (Oberdörster et al. 2005). The smaller particle sizes show an apparent concentration–effect relationship, whereas the larger sizes cause less toxicity (Hund-Rinke and Simon 2006). On the other hand (Fig. 8.3b), the effect concentrations could not be connected to the median values of the size of nanoparticles in media (Warheit et al. 2007). The 140 nm size of nanoparticles and 380 nm in particle diameter in media have the same toxicities to the alga *P. subcapitata* with 72-h EC_{50} of 21 mg/l and 16 mg/l, respectively. Nanoparticles 380 nm in size in media are more toxic than those 1261 nm to the alga *P. subcapitata* (Fig. 8.3b). As well, there is no correlation between the crystalline form of nanoparticles applied in the studies and toxicity for algae (Table 8.2). Studies by some researchers in which particles with the same sizes were tested with the three species of algae observed that the sensitivity to TiO_2 for the alga *P. subcapitata* is more than for *C. reinhardtii*, and *D. subspicatus* is the least sensitive (Aruoja et al. 2009; Hund-Rinke and Simon 2006), although given that some of the variable 72-h EC_{50} values that were obtained for *P. subcapitata* are very high, this cannot be confirmed. It might be possible to evaluate species-specific sensitivity to TiO_2 NPs after more data are acquired on all species with NPs prepared similarly.

No measurements of agglomeration size, which could possibly affect inhibitory impressions, were reported. Effects of Zn and ZnO NPs on metabolic growth in

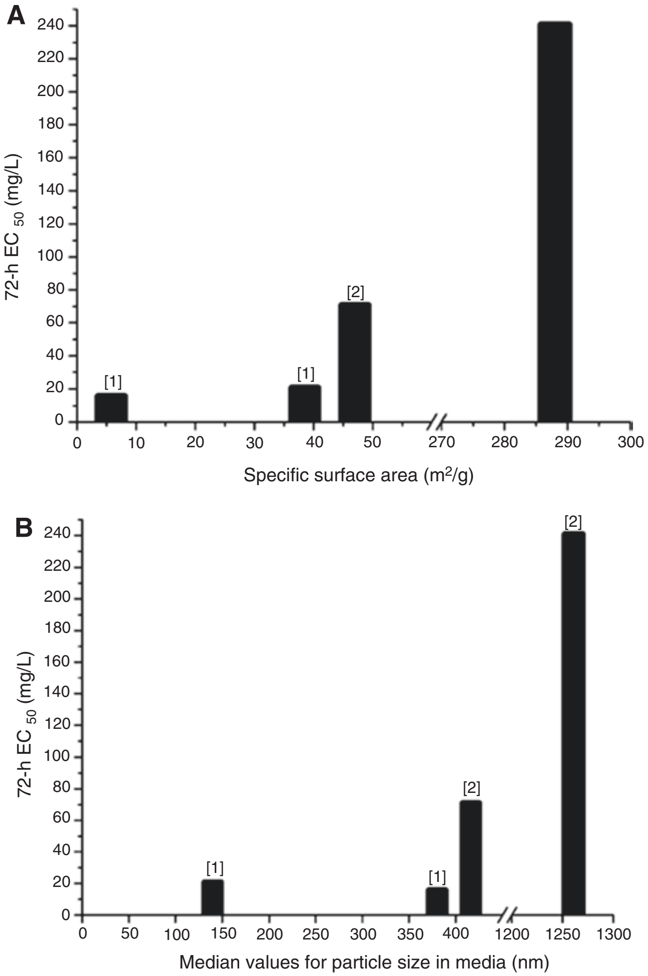

Fig. 8.3 Relationship between (**a**) specific surface area (measured with BET and 72-h EC_{50} values, and (**b**) median values for particle size in media (evaluated with DLS) for TiO_2 NPs for the alga *Pseudokirchneriella subcapitata*. (References: [1] Warheit et al. 2007; [2] Hartmann et al. 2010; Menard et al. 2011)

radish and ryegrass have been shown to be related to their particle size more than their chemical composition (Lin and Xing 2007). At the same time, Al_2O_3 was shown to affect root growth (Yang and Watts 2005). Moreover, silver (Ag) NPs may motivate membrane conversions, increment membrane porosity, and in bacteria cells disrupt their capacity to control transport through the membrane (Pal et al. 2007; Sondi and Salopek-Sondi 2004). Increasing membrane porosity also facilitates the entrance of NPs into cells (Morones et al. 2005). Similarly, it is clear that the properties of materials for nanoparticles differ considerably from bulk materials of the same composition because the increased specific surface area and reactivity

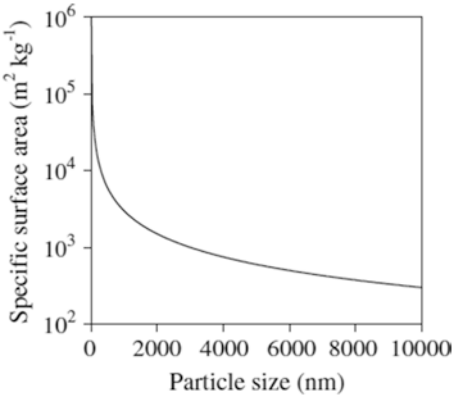

Fig. 8.4 Relation between specific surface area ($m^2\ kg^{-1}$) of a spherical nanoparticle and particle size (diameter in nm) with a density of 1000 kg m^{-3} (Navarro et al. 2008)

Table 8.3 The most studied nanoparticles (NPs) and their physicochemical properties

Type	Diameter (nm)	Specific surface area ($m^2\ g^{-1}$)
Fullerene (C_{60})	0.72	~10,000
Silver (Ag)	10	9–11
Titanium oxide (TiO_2)	5	200–220
Zinc oxide (ZnO)	20	~50
Copper oxide (CuO)	30–50	~13.1
Iron oxide (Fe_2O_3)	20–50	~50
Quantum dots	1–10	100–1000
Single-walled carbon nanotubes	1–2 (diameter) 5–30 μm (length)	~400
Multi-walled carbon nanotubes	<8 (OD)[a] 2–5 (ID)[a] 10–30 μm (length)	~500

From Navarro et al. (2008)

[a] *OD* outside diameter, *ID* inside diameter

may cause increased bioavailability and toxicity (Nel et al. 2006). Indeed, CuO NPs were as much as 50 times more toxic than bulk CuO particles towards algae (Aruoja et al. 2009), crustaceans (Heinlaan et al. 2008), yeast (Kasemets et al. 2009), and protozoa (Mortimer et al. 2010). As well, the antibacterial effects of silver NPs are dependent not only on size (Morones et al. 2005) but also on shape (Pal et al. 2007). The inhibitory effects of Ag NPs to nitrifying organisms are correlated with the sizes in fractions less than 5 nm, which were rather more toxic towards bacteria than their bulk species (Choi and Hu 2008). The indirect effects of NPs are caused mostly by release of toxic ions, for example, metal NPs or the generation of ROS. Furthermore, NPs themselves may act as pollutant carriers, hence enhance or reduce the bioavailability of other toxic matters (Fig. 8.5). Some components present in the environmental matrix might increment NP stability (OM) and thus

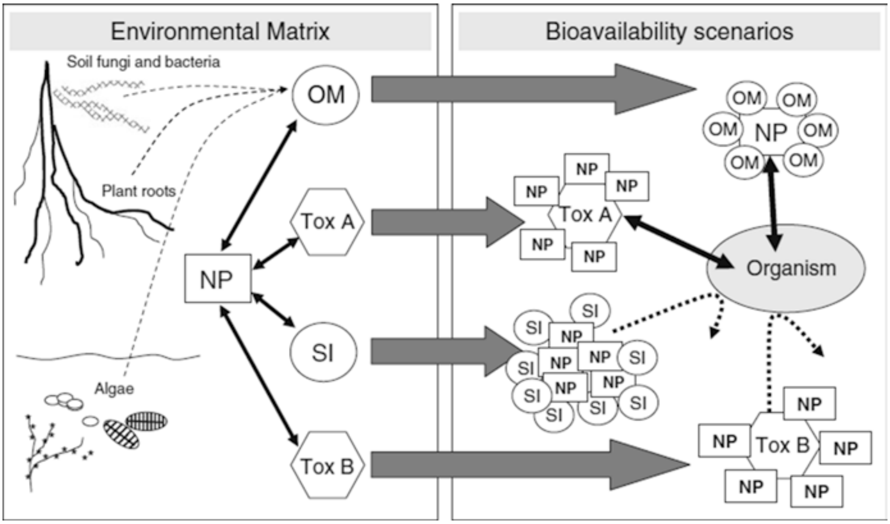

Fig. 8.5 Nanoparticles interactions with toxicants (Tox A and B), salt ions (SI) and organic matters (OM) released by bacteria, plants, algae, and fungi (From Navarro et al. 2008)

bioavailability, whereas others (SI) might cause the aggregation of NPs, hence reducing their bioavailability (rejected organisms are shown as dotted arrows in Fig. 8.5) (Navarro et al. 2008).

As an example of physical abatement, the deposition of NPs on the surface of photosynthetic organisms may prevent photosynthetic activity from shading effects by reducing light availability, and also because of NP toxicity (Fig. 8.6) (Navarro et al. 2008).

As a function of the trend to agglomerate and correlate with other organic matters (Chen and Elimelech 2007), sorption of NPs to algal cells is expected. For example, results show that TiO_2 NPs were adsorbed onto the surface of the algal cell, at a 2.3-fold increment of cellular weight (Huang et al. 2005). Similarly, by aggregation of carbon black, which bound to sperm cells, fertilization success was reduced on *Fucus serratus* (a marine seaweed) (Fernandes et al. 2007). Sorption of greater nanoparticle aggregates to cell walls may also adjust the cellular adsorption of essential nutrients through plugging of the walls or nutrient sorption. In addition, organisms may excrete suspensions as a feedback reaction to alter the NP toxicity. Metal chelate exudation from root systems, for example, siderophores, may decrease the bioavailability of toxic metal ions released from metallic NPs by complexation (Fig. 8.5). Such components may also suppress the dissolution rate of metal from metal oxide NPs or from metallic NPs. Simultaneously, algae can generate substances that may induce metal ion chelation or NP flocculation and cause the bioavailability of both nanoparticle and metal ions thus released to decline (Soldo et al. 2005). Results showed that exopolymeric material production can cause increase in algae at exposure to NPs and may thus cooperate to detoxification reactions (Miao

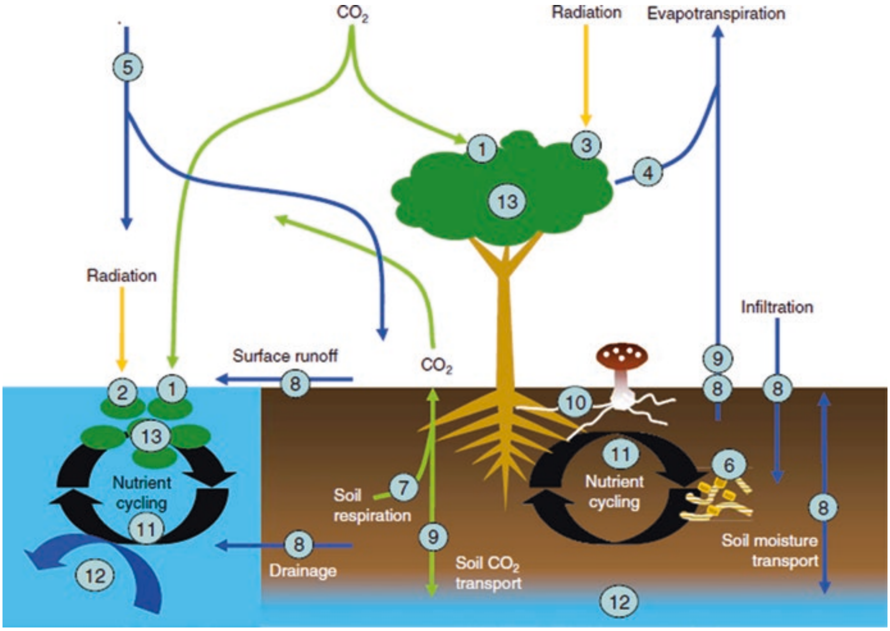

Fig. 8.6 schematic diagram of some aquatic ecosystem processes. Nanoparticles impacts on photosynthetic organisms may decrement the fixation of CO_2 (1); NPs adsorbed (2) or deposited (3) on active surfaces might reduce gas exchange or light availability (4) and hence photosynthesis; NPs in the atmosphere might increase the nuclei available for raindrop formation (5), thus cause to precipitation; NPs' effects on fungi and bacteria(6) might influence on soil respiration (7), and other soil-texture-associated processes such as transport of liquids (8) or gases (9), also modifying symbiotic relationships (10). As well, this might lead to damages in nutrient cycling (11), water purification (12), and biomass production (13). (From Navarro et al. 2008)

et al. 2007). Figure 8.5 shows that bioavailability of NPs can be both improved (Terashima and Nagao 2007) and decreased (Dunphy Guzman et al. 2006) by the abiotic characteristics of the surrounding matrix.

8.6 Generation of Oxidative Stress

Oxidative destruction, caused by either internalized or extracellular NPs, has been discussed as one of the main reasons for nanoparticle toxicity (Nel et al. 2006). Nanoparticles might generate reactive oxidant species (ROS) towards their interaction with organisms or by some agents such as UV radiation that is present in the environment. ROS formation is exactly related to NPs with photocatalytic characteristics such as TiO_2 (Kus et al. 2006). ROS-induced oxidative stress caused some physiological effects at all levels of biological formation. Thus, destructive effects of TiO_2 NPs on bacteria have been improved by sunlight or UV irradiation (Adams et al. 2006). Other NPs, such as fullerenes and silver NPs, also showed the ability to generate ROS by UV exposure (Rodriguez-Moya et al. 2007). Under usual conditions, ROS-induced destruction is adapted by the cellular antioxidant cascade: glutathione peroxidase, catalases, enzymes such as superoxide dismutase (SOD), and non-enzymatic antioxidants, for example, ascorbic acid, α-tocopherol, and glutathione (Blokhina et al. 2003). Currently, Von Moos and Slaveykova (2014) considered various inorganic NPs such as CuO, ZnO, and Ag NPs that induced oxidative stress in aquatic environments. The cellular mechanisms based on oxidative stress and ROS production induced by nanoparticles in aquatic microorganisms and the methods for the evaluation of oxidative stress and ROS have been comprehensively discussed by von Moos and Slaveykova (2014).

8.6.1 Oxidative Stress-Related Effects of Nanoparticles on Relevant Organisms

For evaluation of the NP potential to apply oxidative stress to different environmentally relevant organisms, some biomarkers, such as catalase (CAT), increased activities of SOD, glutathione peroxidase (GPx), increased levels of metallothionein-like proteins and glutathione-*S*-transferase (GST) and have been investigated. There are many examples representing ROS as one of the primary drivers of silver NPs toxic effects. Yet, toxicity of NPs may be mostly the result of their toxicants release (Brunner et al. 2006; Navarro et al. 2008). Ag^+ released from a silver (Ag) NP may inhibit respiratory enzymes and cause induced oxidative stress upon ROS generation (Kim et al. 2007; Aziz et al. 2014, 2015, 2016, 2019). Ag^+ may also link to phosphorus- and sulfur-containing molecules (taurine, *S*-adenosylmethionine, glutathione, cysteine, etc.) involved in cell antioxidant protection (Pappa et al. 2007; Prasad and Swamy 2013), and may result in the reduction of the intracellular concentration of molecules (Hussain et al. 2005). Hence, Ag NPs were found to decrease photosystem II quantum yield, cell-specific growth rate, and chlorophyll *a* capacity of the marine phytoplankton *Thalassiosira weissflogii* and the toxic factors were calculated by the release of Ag^+ (Miao et al. 2007). Similarly, results obtained by Navarro et al. (2007) demonstrated that toxicity of Ag NPs to the photosystem II quantum yield of the freshwater alga *Chlamydomonas reinhardtii* also correlated to the release of Ag^+. Both studies indicate that about 1% of the Ag NPs was released as ionic silver (Ag^+). Moreover, concentrations of Ag^+ were too low to clear the toxicity exhibited by Ag NPs, so recommended the significance of the Ag NP–cell interaction (Navarro et al. 2007; Sotiriou and Pratsinis 2010; Yang et al. 2011). Moreover, by comparing the toxicity of ZnO NPs with that of Zn^{2+}, it was demonstrated that ZnO NP toxicity may depend on the solubility of the NPs (Franklin et al. 2007). Based on recent knowledge, CuO NPs tend to exert more oxidative destruction than Ag NPs. As for Ag NPs, the context of participation of CuO NPs and the dissolved Cu ions to the generation of ROS is still debatable. It is clear that ions of redox-active metals such as Cu may transfer free radicals by the Fenton reaction and deliver intracellular oxidative stress (Valko et al. 2005). The reduction mechanism of cuprous ion [Cu(I)] to cupric ion [Cu(II)] in the presence of biological reducing agents including glutathione (GSH) or ascorbic acid is shown in the following reaction. In this reaction, reactive hydroxyl radicals (OH°) are produced from hydrogen peroxide (H_2O_2) by the Fenton reaction.

$$\mathrm{Cu}\left(I\right)+\mathrm{H_2O_2}\rightarrow\mathrm{Cu}\left(II\right)+{}^{\circ}\,\mathrm{OH}+\mathrm{OH}^{-} \tag{8.1}$$

Induction of ROS by CuO NPs has been presented at different levels of biological constitution. Luminescent bacterial tests have demonstrated ROS-producing potential of aqueous compounds of CuO NPs in recombinant *E. coli* strains (Ivask et al. 2010). Currently, results show that oxidative stress was induced by CuO NPs and DNA damage protection systems in recombinant *E. coli* strains at very low toxic concentrations (0.1 mg Cu/l) (Bondarenko et al. 2012), although these effects were most probably provoked by dissolved Cu ions. Applerot et al. (2012) indicated that upon connection to bacteria, lipid peroxidation and intracellular oxidative stress are induced by CuO NPs. Mortimer et al. (2010) described the effects of CuO NPs on membrane lipids for the unicellular protozoa *Tetrahymena thermophila*: in reaction to exposure to CuO nanoparticles (80 mg/l), the protozoa altered membrane fatty acid suspensions to more hardness by increasing the content of saturated fatty acids (C18:0 and C16:0) and decreasing the content of unsaturated fatty acids (C18:3 *cis*-6,9,12 and C18:2 *cis*-9,12). Peroxidation of lipid caused by CuO nanoparticles has also been discussed in the bacteria *E. coli* and *Bacillus subtilis* (Applerot et al. 2012), the tissue and gills of rainbow trout (Shaw et al. 2012), and the green alga *C. reinhardtii* (Cheloni and Slaveykova 2013). ZnO is a photocatalytic nanoparticle that can promote ROS production equal to or more than its bandgap energy (3.37 eV) that correlates to 368 nm light (Ma et al. 2013). In fact, studies by Yu et al. (2011) and Xiong et al. (2011) demonstrated that under irradiation, at concentrations more than 10 mg/l, ZnO NP components induced hydroxyl radicals ($^{\circ}OH$). Simultaneously, no hydroxyl radical generation was obtained in bulk ZnO particles. Antibacterial properties of ZnO NPs to different bacterial species (Applerot et al. 2009) and yeast (Lipovsky et al. 2011) were generated by reaction of the ZnO NP surface with H_2O, which generates hydroxyl radicals ($^{\circ}OH$). H_2O_2 production was also indicated as one of the main factors participating in the antibacterial activity of ZnO NPs to *E. coli* (Sawai et al. 1998). Particle-induced ROS generation can result in a range of biological reactions, depending on the type of cellular pathways that are activated by oxidative stress and the relative abundance of ROS formation (Xia et al. 2008). Excessive formation of these ROS species can lead to cell membrane damage, which may result in cell lysis and finally cell death (Zhang et al. 2007) or facilitate aggregation and internalization of the NPs into cells, leading to toxicity (Xie et al. 2011). An investigation with the bacteria *Pseudomonas chlororaphis* indicated that ZnO NPs might induce intracellular ROS without illumination (Dimkpa et al. 2011). ROS protection systems have been shown to contribute in the ZnO toxicity reaction of bacteria. Lipid peroxidation caused by ZnO NP-induced ROS has been demonstrated in several organisms such as earthworms (Hu et al. 2010), bacteria (Kumar et al. 2011), and fish (Xiong et al. 2011). By increasing in lipid peroxidation levels because of exposure to ZnO, NPs might represent that the self-scavenging content of antioxidant defense systems was increased (Hao and Chen 2012).

8.7 Photo-Induced Toxicity

A special case of ecotoxicity is given for nanomaterials that have a photocatalytic properties. Ecotoxic effects of these particles might differ from dark and light conditions. For example, the EC_{50} assessed by incubation with TiO_2 for *E. coli* declined from 583 mg/l to 1.68 mg/l when irradiated with sunlight for 30 min (Dasari et al. 2013). An ecotoxic effect of TiO_2 particles as a photocatalytic material has been explained for various organisms, such as bacteria (Dalai et al. 2012), algae (Lee and An 2013), fungi (Lipovsky et al. 2011), and water organisms such as fish (Vevers and Jha 2008) and *Daphnia magna* (Li et al. 2014). In most of the studies, ecotoxicity was related to the concentration of NPs as well as intensity of irradiation. Increased ecotoxicity of CuO NPs (Dasari et al. 2013), ZnO NPs (Lee and An 2013), and QDs[1] (Derfus et al. 2004) was also determined when nanoparticles were irradiated with UV light. In this section, we discuss how irradiation of photocatalytic NPs can influence their toxicity and what observations have been obtained so far.

8.7.1 Photocatalytic ROS generation

Semiconductor nanoparticles have the characteristic that electrons in the valence band are transferred to the conduction band when appropriate energy is absorbed (see Sect. 8.2). The generating electron–hole pair react with water and oxygen to produce ROS. The negative effects from excess ROS inside the cells are described in Sect. 8.6.1. The ROS concentration and their types are intensively dependent on the bandgap energy of the nanoparticles. As we know, nanoparticles with conduction band (CB) energy less than the redox potential of $O_2/O_2^{\bullet-}$ (−0.2 V) participate to generate superoxide radicals, whereas other nanoparticles that provide valence band (VB) energy equal to or higher than the redox potential of $H_2O/{}^{\bullet}OH$ (2.2 V) are expected to improve the generation of hydroxyl radicals, which are the most biologically destructive form of ROS (Miller et al. 2012). Generally, the photocatalytic efficiency of a nanoparticle is evaluated by degradation of a substrate by following detection of the product with different chemical or optical properties. In addition, detection of free radicals by electronic paramagnetic resonance (EPR) is a confirmed method for definition of photocatalytic activity of a nanomaterial and has been recommended in some phototoxicity studies. As for photocatalytic efficiency, the ecotoxic effect has a relationship with particle dosage and light intensity.

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[1] Quantum dots.

Moreover, parameters such as particle size and crystal structure have displayed an influence on phototoxicity. Evidence suggests that the potent TiO_2 P25 NP is more toxic than other TiO_2 NPs form whereas rutile forms show less toxicity than anatase phase particles (Xue et al. 2010). In addition, nanoparticles have a stronger toxic potential for photodegradation than their bulk counterpoints, likely because of their greater surface-to-volume ratio, prompting a higher sorption efficiency of light, although detected photocatalytic activity of a nanoparticle does not necessarily supply phototoxicity. For example, in a study by Tong et al. (2013), rutile TiO_2 NPs degraded methylene blue (MB) but did not cause any toxicity to *E. coli*. Evaluation of an ecotoxic hazard towards organisms and cells has been carried out in vivo and in vitro. In case of vitro tests, traditional methods are adapted and an irradiation light source is contained as an additional parameter. To allow a logical conclusion, it is essential to maintain truly identical conditions during both conditions (dark and light) and to make known appropriate negative controls that demonstrate the effect of light or nanoparticles on the cells. Moreover, in common cytotoxicity tests, exactly the production of ROS and their effect on cellular matters are considered, as this is assumed to be the main cause of photo-induced cell death. ROS levels were in fact significantly increased when cells or organisms were similarly exposed to both TiO_2 nanoparticles and light irradiation. The ecotoxic effect has been derived from improved GSH depletion, increased SOD activity, or lipid peroxidation (Dasari et al. 2013). In addition, improved LDH[2] release is mostly taken as an indicator for ecotoxicity (Rozhkova et al. 2009). The identification and detection of ROS might be applied in both the absence or in the presence of cells. Commonly, the ROS levels can be tested by detection of fluorescent dyes such as H_2DCFDA. As H_2DCFDA does not recognize different radicals but is reduced by some reactive species that present in the cells, the hydroxyl radical sensitive dye (APF) is increasingly applied (Friehs et al. 2016). The generation of hydroxyl radical has been obtained by APF conversion for ZnO and TiO_2 NPs in *Daphnia magna*, *E. coli*, and HeLa cells (Friehs et al. 2016). The production of ROS by photocatalytic NPs such as TiO_2 and several effects observed on cellular members, as well as the mechanisms considered for cellular destruction, are depicted in Fig. 8.7.

Characterization of the radical species has also been applied by some scavenging tests that give some information on the radical formation of irradiated TiO_2 particles in cellular systems. Many scavengers that are applied indicate a selectivity towards a radical species. Evidence from its cellular function shows SOD can be applied as a specific superoxide anion scavenger, whereas catalase converts hydrogen peroxide (Friehs et al. 2016).

Natural materials have been obtained for quenching of $^{\bullet}OH$ radicals: mannitol (Rozhkova et al. 2009), or L-tryptophan (Cai et al. 1992), or H_2O_2 (sodium pyruvate). Scavengers for cell-free systems are DMSO[3] ($^{\bullet}OH$), sodium azide ($^1O_2$ and

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[2] Lactate dehydrogenase.

[3] Dimethyl sulfoxide.

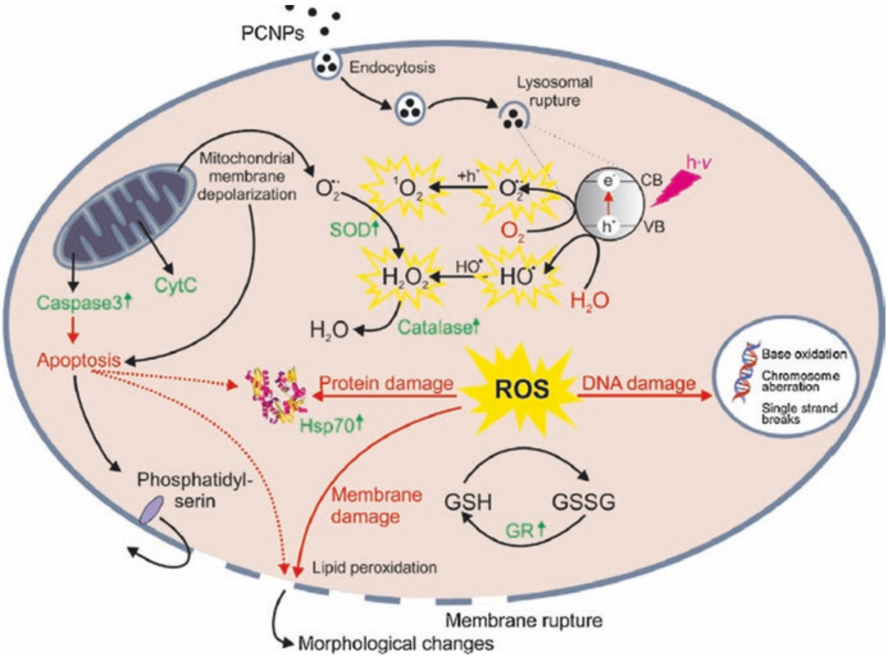

Fig. 8.7 ROS generation by photocatalytic NPs and the effects on cellular members. (From Friehs et al. 2016)

•OH), and dyes such as singlet oxygen sensor green dye. In addition to SOD, Tiron[4] has been applied to detect superoxide radicals (Idris et al. 2014).

Moreover, Fenoglio and coworkers (2009) explained a mechanism involving the electron transfer (e⁻) to an acceptor compound, that is, dissolved oxygen, to generate superoxide anion. The carbon-centered free radicals thus produced can initiate the multistep reactions that cause oxidative destruction of biological molecules such as lipids or nucleic acids (Dalai et al. 2012). Other studies supposed the ROS generation under dark conditions to be carried out by extracellular sorption of nano TiO_2 inducing intracellular ROS production (Dalai et al. 2012). Lakshmi Prasanna and Vijayaraghavan (2015) reported another mechanism for generation of ROS from ZnO nanoparticles in dark conditions. They confirmed the EPR studies of ZnO NP in dark condition in the presence of singly ionized oxygen vacancy that induced the oxygen reaction from the atmosphere with an electron from the ZnO NP surface to generate a superoxide and hydroxyl radicals, respectively (Eqs. 8.2, 8.3, 8.4, and 8.5):

$$O_2 + e^- \rightarrow O_2^{\bullet -} \tag{8.2}$$

$$O_2^{\bullet -} + H_2O \rightarrow {}^{\bullet}HO_2 + OH^- \tag{8.3}$$

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[4] Disodium 4,5-dihydroxy-1,3-benzenedisulfonate.

$$^{\bullet}HO_2 + {}^{\bullet}HO_2 \rightarrow H_2O_2 + O_2 \quad (8.4)$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + {}^{\bullet}HO + OH^- \quad (8.5)$$

Moreover, ROS production is supposed to be the main inductor of improved cell death by the irradiated photocatalytic NPs, although induction of oxidative stress does not always relate to photocatalytic activity of a nanoparticle. Some researchers (Dasari et al. 2013) found improved LDH release and GSH depletion in *E. coli* for CuO nanoparticles under irradiation conditions, but no improved ROS generation. Thus, other effects towards photocatalytic degradation of cellular compounds might cause light-induced toxicity. Lipovsky et al. (2011) indicated that smaller ZnO NPs inhibit the growth of the fungus *Candida albicans* more than larger particles. Here, enhancement in toxicity was correlated with a more possible dissolution of Zn^{2+} ions from smaller sizes. In case of unstable particles such as CuO, ZnO, or CdSe, simultaneous irradiation caused increased release of free ions, resulting in higher toxicity. ZnO undergoes dissolution either in acidic conditions or under UV radiation, as shown by the following reaction mechanisms (Han et al. 2010):

$$ZnO + 2H^+ \rightarrow Zn^{2+} + H_2O \quad \text{Dark dissolution}\left(\text{acidic condition}\right) \quad (8.6)$$

$$ZnO + h^+ + H^+ \rightarrow 1/2H_2O_2 + Zn^{2+} \quad \text{Photo-corrosion}\left(\text{UV radiation}\right) \quad (8.7)$$

The photo-generated holes on ZnO NP surfaces can migrate the ZnO bond and finally oxidize ZnO to Zn^{2+}. Simultaneously, CdSe suffers dissolution reactions, when the photo-generated holes release Cd^{2+} from CdSe (Xi et al. 2011). The released ions can produce ROS by the Fenton reaction or damage cellular pathways (Rahman et al. 2013).

8.8 Conclusion

The environmental use of nanosized particles and their inadvertent release exposes aquatic plants, algae, and fungi to these materials. However, there is considerably less information on several key aspects, which prevents a better evaluation of the ecotoxicity of NPs to these ecosystem organisms. According to the unique physicochemical characteristics of nanoparticles, hazard assessment is a challenge, and NP–cell interactions are not fully known. Several challenging questions are still unanswered: (a) the related nanoparticle concentrations in aquatic and aerial environments, (b) the physicochemical properties of the NPs and exposure routes, (c) the specific properties that are correlated to the ecotoxicity effects of NPs, and (d) the mechanisms permitting NPs to pass through cell walls and cellular membranes. For particles that are applied in the field of photocatalysis, explanation of the ecotoxic mechanisms for photocatalytic NPs as well as stability of the evaluations are essential factors to investigate the potential ecotoxic effects referring to characterization of materials. This chapter indicated that the consideration of light irradiation in ecotoxicity tests of photocatalytic NPs has in fact an outcome for endpoint assessment for many organisms that is not negligible. On the other hand, evidence shows that even agglomerated NPs can be more toxic because of their solubility or other specific characterization. Databases based on these ecotoxicity tests may contribute to clarify some of the knowledge concerning photocatalytic NP–cell interactions to increase the quality of ecotoxicity studies.

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Chapter 9
Nanotoxicity: Sources and Effects on Environment

Angana Sarkar, Debapriya Sarkar, and Kasturi Poddar

9.1 Introduction

Particles having size between 1 and 100 nm are termed as nanoparticles (NPs). They consist of a core which is generally inactive or inert in nature with a surface stabilizer which provides them with specific surface physiology and chemistry that make them appropriate for their particular use. Both the core and the surface stabilizer contribute to the biological sensitivity. Their smaller size, easy reproducibility, and higher activity make them an attractive ingredient of modern-day products (Batista et al. 2015). By 2005, the number of products available in the market containing nanoparticles was almost 54, and it reached to 1015 by 2010 (Wilson 2010). In the present market, the availability of such products is much higher in number. With these increased number of available products and usage, the rate of nano-pollution production and quantity of nanowaste deposition has increased significantly. Nanowaste can be classified as engineered nanowaste, which is man-made and is not naturally found in the environment, and natural nanowaste, which is mainly a product of volcanic eruptions, forest fires, lightning, etc. (Tiede et al. 2008). Nanowaste has different kinds of sources, like, release through vehicular combustion, release from industrial processes or through industrial products like toothpaste, biological solid particles from waste water treatment plant that are used in agricultural fields, consumer products production and usage like cosmetics, pesticides, air or water filter etc. (Hou et al. 2013; Sangeetha et al. 2017).

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9.2 Toxicity of Nanomaterials on the Environment

Toxicity of any nanoparticle to living cells is relative to their various parameters, like shape, size, concentration applied, surface chemistry of the nanoparticles, and duration of its exposure to living cells. To determine the different reasons responsible for release of toxic elements in the environment, researchers have studied various industrial production systems that have the potential to release out nanoparticles as by-products of production systems. Various studies, assumptions, and interpretations were made, and clinical tests on animal models were performed to determine the biocompatibility and toxicity potential of the different compounds constituted in the nanoparticles (Table 9.1). However, the different causes noted for toxicity by Kirchner et al. (2005) are mainly:

I. Toxic composites that constitute the nanoparticle which is observed in cadmium selenide nanoparticles where Cd^{2+} ions are released.
II. The small size of nanoparticles contributes to the easy attachment with the cells and its penetration potential. These enable rapid interaction of nanoparticles with other biomolecules within the cell membrane (Khalili Fard et al. 2015). The shape of the nanoparticles enhances its internalization within the cell membrane and triggers toxic reactions.
III. The reactive oxygen species and free radicals formed through high reactivity of nanoparticles oxidize different cellular enzymatic pathways (Fig. 9.1). This oxidative stress is generally exerted through.
IV. Production of oxidant molecules by the nanoparticles themselves or through stimulation of ROS generation as cellular response

    - Transition metal contaminants or transition metal-based nanoparticles that are used as catalysts in the production of different nonmetal nanoparticles
    - Presence and activation of redox groups produced from functionalization of the nanoparticles
    - Presence of free radical intermediates on the surfaces of reactive nanoparticles (Khalili Fard et al. 2015)

The different nanoparticles and their mechanism of cytotoxicity have been enlisted in Table 9.1.

9.3 Bioaccumulation and Biomagnification

Nanoparticles get accumulated in the single-celled organisms through surface interaction. Most of the nanoparticles can easily penetrate through the cell wall rapidly by the virtue of their small size and surface chemistry. This process is called bioconcentration (Arnot and Gobas 2006). It does not include uptake of pollutants via food. Bioaccumulation is a process in which contaminants are received by the body of an organism via different paths, including the food, in a higher rate than the

Table 9.1 List of nanoparticles with their cytotoxicity mechanism

Nanomaterials	Composition	Mechanism of toxicity	References
Metallic	Gold	Size-dependent cytotoxicity. Larger, anisotropic AuNPs have more exposed surface available for oxidation and are cytotoxic than the smaller ones. Associated cationic side chains, surface-coated ligands, and stabilizers used with AuNPs also increase its cytotoxic effects. Higher surface area-to-volume ratio provides higher surface activity	Yildirimer et al. (2011); Yah (2013); Khalili Fard et al. (2015)
	Silver	Accumulation of significant amount of AgNPs exhibits toxicity through oxidation. These particles then degrade the antioxidant cells in our body. ROS generation in also higher from AgNP compared to bulk silver. Size and coatings provided over AgNPs also produce cytotoxic effects	Yildirimer et al. (2011); Bahadar et al. (2016); Khalili Fard et al. (2015)
Metallic oxide	Aluminum oxide (Al_2O_3)	Al_2O_3 NPs have the ability to oxidize cells and inhibit respiration and permeability of cells leading to cell death	Arul Prakash et al. (2011); Srikanth et al. (2015)
	Copper oxide (CuO)	CuONPs reduce glutathione and increase lipid peroxidase, catalase, and superoxide dismutase in the epidermal cells of humans	Alarifi et al. (2013)
	Zinc oxide (ZnO)	Generates reactive oxygen species and helps in the disintegration of cell membrane which reduces cellular viability. It alters the cell cycle with micronuclei production, phosphorylates the H2AX molecules, and produces DNA damage	Valdiglesias et al. (2013)
	Iron oxide (Fe_2O_3, Fe_3O_4)	Lower concentration does not have any report with adverse effect but with higher concentration of accumulation can result in imbalance in its homeostasis and can cause aberrant cellular responses including cytotoxicity, DNA damage, oxidative stress, epigenetic events, and inflammatory processes	Singh et al. (2010)
	Titanium oxide (TiO_2)	The mechanism is not yet clear. These nanoparticles do not release metallic ions, but they interact with different biomolecules present inside through surface to surface interaction. This leads to generation of ROS, mitochondrial depolarization, plasma membrane leakage, intracellular calcium influx, and cytokine release. They also possess photocytotoxicity	Xiong et al. (2013); Ghosh et al. (2013)

(continued)

Table 9.1 (continued)

Nanomaterials	Composition	Mechanism of toxicity	References
Non-metallic	Carbon nanotubes	Multiwalled carbon nanotubes are able to stimulate the release of cytokines, IL-1β, TNF-α, IL-6, and IL-8 from mesothelial cells and macrophages. They induce ROS generation in the several cell lines and activate different ROS-associated intracellular signaling pathways such as mitogen-activated protein kinase (MAPK), activator protein-1 (AP-1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in mesothelial cells	Murphy et al. (2011); Johnston et al. (2010); Clift et al. (2014)
	Quantum dots	The Cd^{2+} ions present in QD structures are often found to interfere with the DNA repair process. It even substitutes the physiological Zn content. Cd^{2+} readily helps in the oxidation of CdSe- and CdTe-derived QDs and achieves photoactivation on exposure to UV or visible light	Khalili Fard et al. (2015)
	Silica	Induces cell death with long-term exposure and lethal dose applied. Enhances immunotoxicity with the gene expression activation and release of interleukins IL6 and IL8. Activates reactive oxygen species. Increases malondialdehyde levels and upregulates pro-apoptotic genes, caspase-3and p53, leading to autophagy	McCarthy et al. (2012); Murugadoss et al. (2017)

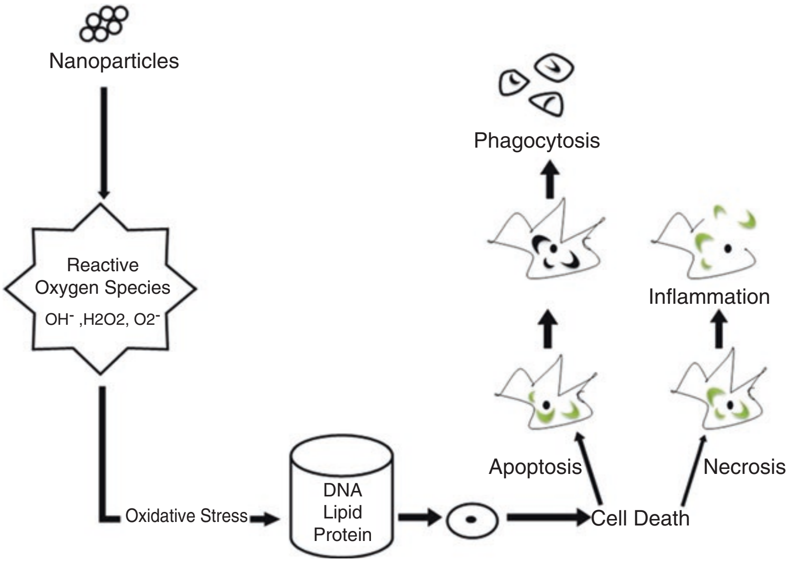

Fig. 9.1 ROS generation from nanoparticles and its cytotoxic effects

same being excreted out or degraded within the body (Stadnicka et al. 2012) (Fig. 9.2). As a result of this, the amount of a particular pollutant raised gradually. When organisms with bioaccumulation of a particular pollutant is eaten by other organisms higher in the food chain, they acquire the pollutants in a higher dose that exhibits the worst effect. This process is called biomagnification. These processes were previously reported with pollutant like POP (persistent organic pollutant), for example, dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene, etc. The same mechanism has been observed in case of different nanoparticles and the effects of it can be seen in organisms present in higher trophic levels (Gray 2002).

Bioaccumulation property of any nanoparticle can be expressed as bioaccumulation factor (BAF) or bioconcentration factor (BCF) which is a ratio of concentration of a particular nanoparticle in an organism to the same in water. BAF depends on different factors which include the type of nanoparticle, size, concentration, exposure time, and the organism. BAF of different nanoparticles has been reported by different researchers. Zhu et al. (2010) have reported that TiO_2 nanoparticle of size 21 nm, which eventually aggregates and forms particles of 3500 nm, on exposure for 24 h (aqueous solution of 0.1–1 mg/l, pH = 8) to *Daphnia magna* resulting in a bioconcentration factor (BCF) of 56,563 for 0.1 mg/L TiO_2 and 118,063 for 1 mg/L TiO_2. These data were obtained without providing any diet to the organism. The same experiment when repeated along with a diet for the organism, showed a reduce in BCF to 1232 at 1 mg/L TiO_2.

Zhao and Wang (2010) conducted a similar experiment on *D. magna* where they selected radiolabeled cysteine coated AgNP with particle size of 10 nm which were aggregated to form 40–50 nm size particles. The organism was exposed to a water solution of 500 µg/L nanoparticle. They reported the uptake of ionic Ag to be higher than that of AgNP and 70% of the ingested AgNP were accumulated (Zhao

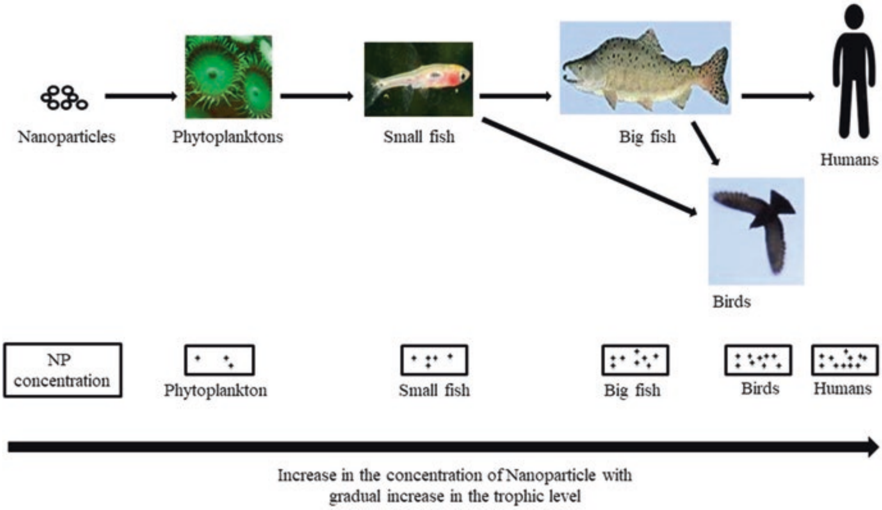

Fig. 9.2 Bioaccumulation of nanoparticle concentration with an increase in the trophic level

and Wang 2010). They further continued their investigation and found the BCF of 46,000 for 0.5 mg/L AgNP with an incubation time of 48 h (Zhao and Wang 2011).

Gaiser reported that exposure of 0.01–0.1 mg/L aqueous solution of AgNP with a primary size of 35 nm to *Cyprius carpio* showed the accumulation of AgNP in liver, bile, intestine, and gills. These reports are showing that different nanoparticles have great capability of bioaccumulation. These bioaccumulated nanoparticles have a tendency to show a biomagnified effect in higher trophic-level organisms (Gaiser et al. 2011).

9.4 Potential Sources of Contamination of Different Nanoparticles

With the development of nanotechnology, extensive study over different nanoparticles opens up a wide range of implications of compounds in our daily life. Extensive use of such nanoparticles in the present day has increased the unwanted deposition of nanoparticles in the environment. This section contains the probable contamination sources of different nanoparticles.

9.4.1 Metallic Nanoparticles

9.4.1.1 Silver NP

It contains a core made of silver of 1–100 nm size. Due to their large surface to volume ratio, they are highly active and are often present as silver oxide. They usually contain numerous types of ligands according to their use. Silver NPs are used in various fields, for instance, they are often used as antimicrobial substance (Yamanaka et al. 2005; Prasad 2014; Aziz et al. 2014, 2015; Joshi et al. 2018), vehicle for chemotherapeutic drug (Ock et al. 2012), etc. They are also used as catalysts to reduce dyes, selective oxidation of benzene, and synergistic oxidation of carbon dioxide (Jiang et al. 2005). The antimicrobial activity of silver nanoparticle has drawn great attention of different researchers and inventors as they found its activity to be more efficient than any other commonly known antimicrobial substances and is cost-effective too. Due to this, they are being used to sterilize surgical instruments. The surfaces of surgical instruments are generally provided with a coating of silver nanoparticles (Jo et al. 2014; Aziz et al. 2016). The same property of AgNP makes them useful for textile industry where they are used in clothings to reduce the order of sweating (Mueller and Nowack 2008). But the problem is that after washing, most of these NPs are washed out of the fabric and get deposited in the aquatic system. Apart from these, AgNP contamination also occurs through wastewater treatment plants, pesticides, laundry waste and electronic instruments like washing machine, dishwasher, etc.

9.4.1.2 Titanium Oxide

Titanium oxide nanoparticle is one of the most advertised nanoparticles. It is often referred as the "legacy nanoparticle" (USNIOSH 2016). Due to its photo-absorbing ability, it is used in UV protection in sunscreens, windshield, paint, tiles, and anti-fogging agents. Engineered TiO_2 nanoparticles have been successfully and efficiently used in light-emitting diodes and solar cells. More commercialized use of TiO_2 nanoparticles is in UV protection glass and other accessories (Environmental Working Group 2017). The problem is same as that of AgNP. They tend to get washed out from the surface where they are integrated and eventually get accumulated in aquatic systems. Apart from these, they also get accumulated in the soil. They also have the tendency to get bioaccumulated and biomagnified. With their photocatalytic activity, they are very harmful to the microflora and fauna of our environment. However, the various environment-governing institutions have not yet been able to take any safety measures against this nanoparticle.

9.4.1.3 Copper Oxide

Copper nanoparticles are widely used in different sensing devices, such as glucose sensor, amino acid detector, etc. It is useful for various applications due to its high catalytic activity in a wide range of pH and temperature (Ibupoto et al. 2013). Apart from these, it is used in different electronic devices due to its thermostability and high conductivity. But when these devices are disposed off as electronic wastes, these nanoparticles tend to get accumulated in the soil and aquatic systems. With every passing day, the amount of such wastes generated in the environment gradually increases. CuNP also has the ability of bioaccumulation and biomagnification in the environment.

9.4.1.4 Aluminum Oxide

Aluminum oxide nanoparticles or alumina nanoparticles are widely used in different industrial purposes, mainly in downstreaming. The most renowned uses of alumina-NP are obtained as an adsorbent to adsorb hydrocarbon impurities from air, brightening solution in sugar production, vapor recovery of hydrogen fluoride, etc. It is also being used as a drying agent or desiccator to dry gases. It is also useful for the protection of different instruments from humid air. It is also used in the treatment of wastewater of nuclear power plants as a sorbent of radionuclides. Alumina-NP also gains importance in the polishing industry. From all the wide range of its usage, aluminum oxide nanoparticles tend to get contaminated in the soil and aquatic life. As their size is very small, it is very hard to identify their leakage or loss. They have the tendency of bioaccumulation and biomagnification as it has been reported that AlNP moves from a lower trophic level to a subsequently higher trophic level through the food chain (Sadiq et al. 2011).

9.4.2 Nonmetallic Nanoparticles

9.4.2.1 Silicon

Silicon nanoparticles are one of the most widely used nanoparticles. They are mainly used in luminous and display devices, micro and integrated semiconductors, solar energy cells, and lithium-ion batteries. Apart from these, they are modified into silicon nanotubes, nanowires, and nanofibers which are used in many modern sophisticated devices (AZoNano (Si) 2013). Nanoparticles made of silicon dioxide or silica are also used in various fields. They are mainly used in rubber and plastic industries. They are used to straighten concrete and other construction composites. They are widely researched elements for use as potential vectors for drugs and other therapeutics (AZoNano (SiO_2) 2013). When the electronic devices are disposed off, the electronic waste tends to degrade and release silicon in the environment. It acts as a source from which the silicon radiates out and contaminates the nearby soil and aquatic ecosystem.

9.4.2.2 Carbon Nanotubes

Due to their structural integrity and toughness, carbon nanotubes are widely used for building different structures, like wind turbine, handle bars, forks, and aero bars. Due to their lightweight, they gained attraction for different sports gears like baseball bat, hunting arrows, surf boards, ice hockey sticks, skies, etc. (Pagni 2010). However, when it comes to environmental contamination of carbon nanotube, the main source is the burning of different fossil fuels and other natural events such as volcanic eruption, forest fire, etc. It has been reported that multiwalled carbon nanotubes tend to get self-aggregated in the exhaust gas from different combustion. Unlike other nanoparticles, the pollution contributed by carbon nanotubes is mainly captured in the air which is significantly severe than the pollution produced by other nanomaterials in soil or aquatic systems (Murr et al. 2004).

Till now, there is not much concern about pollution related to nanoparticle or “nano-pollution”. The mentioned nanoparticles have been identified with some significant contamination level. Other than these nanoparticles, there are certain other nanomaterials which are suspected to have the possibility of contamination, which include gold nanoparticles, zinc oxide nanoparticles, ferromagnetic nanoparticles, and quantum dots. Judy et al. have already reported the biomagnification of gold nanoparticle in terrestrial food chain (Judy et al. 2011).

9.5 Conclusion

Nanoparticle with their smaller size and higher surface to volume ratio expressed higher reactivity than their bulk materials. Due to this, the toxicity and reactiveness of nanoparticles differ from the bulk material. Bulk aluminum, gold, silver, and titanium are known as most stable and inert metals with respect to biological activity. However, when these metals constitute nanoparticles, they become cytotoxic due to their improved reactivity, as discussed in Table 9.1. Bioaccumulation of these nanoparticles makes this worse. Due to bioaccumulation, these nanoparticles get concentrated inside the organism's body. It starts with the lowest trophic-level organisms like planktons, algae, etc. and shows its biomagnified effect on the highest trophic-level organisms like, birds, beers, humans, etc. With the development and advancement in nanotechnology, products with nanoparticles are getting great success in the market, with an increasing demand. This results in more events of environmental contamination with nanoparticles. Different nanoparticles, mainly AgNP, TiO_2NP, SiNPs, etc., are getting deposited in soil and aquatic systems. Their presence in soil hinders the normal and healthy soil ecosystem due to the antimicrobial activities of these nanoparticles. On the other hand, aquatic planktons accumulate these nanoparticles by the process of bioconcentration and by transferring them to higher trophic-level organisms by the process of bioaccumulation and biomagnification. Thus, the aquatic life is affected greatly with this pollution. Pollution related to nanoparticle or nano-pollution is gaining a lot of attention recently. To avoid such pollution, certain measures need to be taken to prevent the washout of nanoparticles which is one of the main reasons for nanoparticle deposition in the soil and aquatic systems. The devices or instruments containing different nanoparticles must be disposed safely and securely so that nanoparticle deposition in the environment can be avoided. In general, nanoparticles have certainly high half-life which suggests that it could be possible to recycle the nanoparticles of these disposed devices and instruments. Apart from this, the detection procedure of nanoparticle contamination must be improved for better monitoring the soil and aquatic systems.

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Chapter 10
Role of Nanomaterials in Food Preservation

Chandan Ghosh, Debabrata Bera, and Lakshmishri Roy

10.1 Introduction

Foods are composed of carbohydrates, proteins, fats, fiber, vitamins, minerals, and water, thus making them more susceptible to microbial and chemical changes. Microbial contamination of food is often via air, water, soil, and raw materials used as food. Microbial enzymes secreted during their multiplication split complex components like carbohydrate, protein, and fat to different undesirable products like various organic acids, amino acids, H_2S, NH_3, and free fatty acids. These chemical/enzymatic reactions change the color, flavor, texture, taste, nutrient value, and shelf life of the food products. Food quality is also affected in the presence of light, oxygen, etc. Rancidity is an outcome of similar hydrolytic reaction of fats. Unsaturated fat is oxidized to peroxide compounds in the presence of oxygen and oxidizing agents. This phenomenon is restricted in the presence of antioxidant, e-donating agents, and reducing agents.

Some of the spoilage-causing microbes may be pathogenic, which produce different toxins or cellular components detrimental to human health. Thus an effective strategy of food preservation could be by impeding microbial contamination and their action. Microorganisms may be eliminated by different physical and chemical methods. Best physical method is heat treatment. But excessive heat may change the nutritional quality of the foods. So, recently hurdle technology is used to combat this problem. Different selective nanoparticles are able to kill the microorganisms

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and have reducing properties, thereby demonstrating the plausibility of their application for preservation of the quality of food. Hence nanoparticles may be used for preservation of foods.

Nanotechnology in food industry is a less-experimented area. Few studies that have already been carried out illustrate that nanotechnology has a potential impact on food system, particularly in food safety, molecular synthesis of food products, etc. As nanomaterials exhibit phenomena different from their bulk counterpart depending on their size and shape, so use of nanomaterials in the food chain impedes size- and shape-guided biological processes on food systems, particularly in processed food products and food packaging. Briefly, ingredients of living organisms such as proteins, DNA, amino acids, hormones, etc., consist of hierarchical organization of atoms and molecules at nanoscale; hence their tuning or modification becomes easy by nanomaterials due to dimension matching.

Presently, nanomaterials of metal (e.g., Ag, Fe, Ca, Se, etc.), metal oxides (viz., ZnO, MgO, TiO_2, CeO_2, etc.), polymer (chitosan, polypyrrole, polyamide, etc.), and organic materials (lipid, protein, carbohydrate, etc.) are being used in different food sections. In short, it may be stated that nanotechnology opens the door of new array of next-generation food products having good quality and high security. For example, fresh fruit, vegetables, meat, poultry products, and fish seem to be potential vehicles for transmission of pathogenic microbes that leads to foodborne diseases. Here, nanomaterials have rescued us due to their antimicrobial activity. Suitable nanomaterials are either mixed directly to foods or used in packaging system.

Utility of nanotechnology as a tool in the food industry includes (1) increased security of manufacturing, processing, and shipping of food products through sensors for pathogen and contaminant detection, (2) devices to maintain historical environmental records of a particular product and tracking of individual shipments, (3) systems that provide integration of sensing, localization, reporting, and remote control of food products (smart/intelligent systems) and that can increase efficacy and security of food processing and transportation, and (4) encapsulation and delivery systems that carry, protect, and deliver functional food ingredients to their specific site of action. Nanodispersions and nanocapsules are available for delivery of the functional ingredients of drugs, vitamins, antimicrobials, antioxidants, flavorings, colorants, and preservatives. Association colloids, i.e., surfactant micelles, vesicles, bilayers, reverse micelles, and liquid crystals, are used to encapsulate and deliver polar, nonpolar, and/or amphiphilic functional ingredients as depicted in Figs. 10.1 and 10.2, respectively. Nanotechnology is directed for food applications by two different approaches as "bottom up" (deals with building and growing of larger structures from atoms and molecules) and "top down" (is based on a physical processing of the food materials).

10.2 Nanotechnology in Food Quality

Nanotechnology is being significantly used to improve food qualities such as color, flavor, nutrition value, and flow character or to reduce detrimental effect of excess fat, sugar, salt, etc., of the food products.

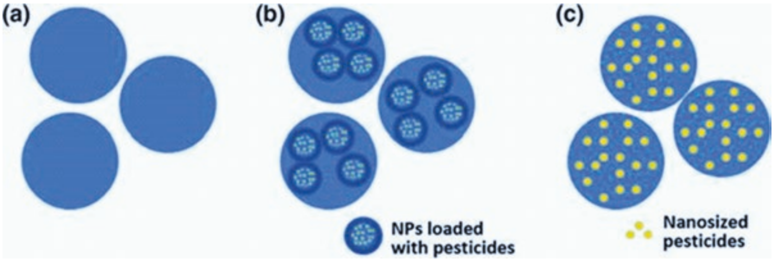


Fig. 10.1 Types of nano-loaded products

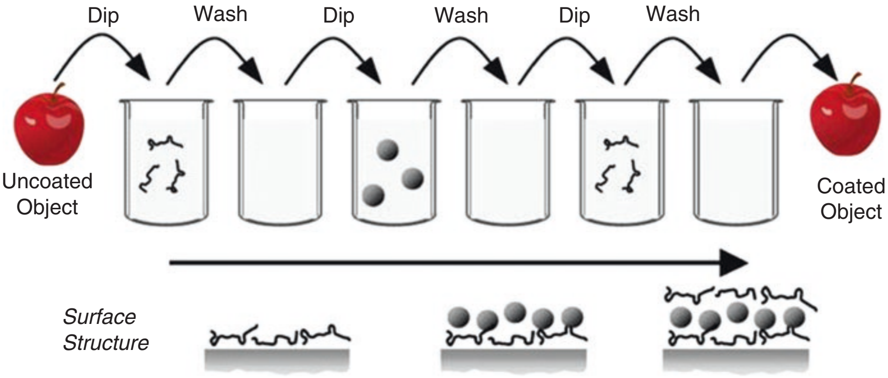


Fig. 10.2 Formulation of nanocoated products

- As an example, triacylglycerol immobilization onto functionalized SiO_2 nanoparticles has been observed to hydrolyze olive oil; hence adaptation and stability of the food get increased significantly (Bai et al. 2006).
- Ceramic nanoparticles exhibit catalytic activity that limits thermal polymerization of oil and results in crisper deep-fried food with longer shelf life.
- Often, enzymes are used to improve flavor and nutritional quality of food. Few enzymes are insoluble in water; hence their effects get decreased due to nonuniform dispersion in food matrix. However, nanotechnology has offered certain nanomaterials on which enzymes can be immobilized, thereby increasing dispersion in food matrix and enhancing enzyme activity.
- Nano-supplements, nano-additives, and nano-delivery system are other important developments of nanotechnology to improve quality of the food products. For example, nano-supplements, added to food stuffs, increase taste and texture of food products along with minimization of fat of the food, yielding healthier food.
- Nano-textured mayonnaise, spread over ice cream as nano-supplement, reduces fat while imparts creaminess of the ice cream.

- The use of beta-carotenes and phytosterol functionalized nanoparticles in food system helps to prevent cholesterol accumulation (Mozafari et al. 2008). Additives of Ag, Fe, Mg, Ca, Se, etc., used for coloration and preservation or to increase flavor and taste, are often found to be insoluble or not well dispersed in water in macroscopic form; thus their efficiency gets reduced. To increase dispersion, functionalization with surfactant or emulsification is required. Alternative is synthesis in nano-dimension, wherein dispersion increases and consequently activity gets enhanced.
- Functional ingredients like vitamins, antioxidants, flavors, preservatives, bioactive peptides, probiotics, etc., offer improvement of quality of processed foods. They are used as a part of a delivery system, rather than in pure form, that cause instability under processing conditions (temperature, moisture, gases (O_2, CO_2, etc.), light, pH, etc.). Generally, ingredients are classified according to their molecular and physical forms such as polarities (amphiphilic/polar/nonpolar), molecular weights (low/high), physical states (gas, liquid, solid), etc., and depending on biochemical characteristics of these ingredients, several strategies like immobilization, nanodispersion, association colloids, and nanoencapsulation have been developed to protect them from harsh chemical or biological environment causing degradation and to deliver them to desired sites keeping taste and texture the same.
- Nano-colloidal strategy is adopted for encapsulation and delivery of polar, nonpolar, and amphiphilic ingredients.
- Nano-micelle process is employed to encapsulate nonpolar bioactive ingredients like lipid, vitamin (A, C, E, Q_{10}, etc.), antioxidant, flavorants, etc.
- Sustained release of ingredient at target sites is another feature of delivery system that has to be considered for the development of efficient delivery system wherein sustained release is achieved on the basis of biological environment like pH, temperature, etc. Different biopolymers and copolymers are used here. For example, cochleate which is a multilayer structure of lipid bilayer in spiral form can encapsulate bioactive materials with poor water solubility, hydrophilic molecules, drug, peptide, protein, etc., and exhibits its strong potency in nanoscale. Biopolymer matrices like chitosan nanoparticles and nanofibers show potency for this purpose. Nowadays, graphene and metal chalcogenide nanoparticles are used as durable and efficient carrier of antioxidants, antibrowning agents, and enzymes, to extend shelf life (Vargas et al. 2008).
- Lipid, protein, and carbohydrate nanoparticles in next-generation delivery system.

Very recently, it has been investigated that nanomaterials of lipid, protein, and carbohydrate can be used to develop efficient delivery system of food ingredient. Main advantage lies with easy digestion in gastrointestinal tract. For example, nano-colloidal delivery system has been developed using lipid nanoparticles for hydrophobic bioactive ingredients of color, flavor, nutrients, nutraceuticals, antioxidant, etc. The major advantage of using lipid nanoparticles is that they also act as source of functional ingredient, thus helping to improve delivery process. There exist few optically transparent lipid nanoparticles, desirable for clear food products and beverage. Lipids, used here, are neutral lipids such as triacylglycerol, diacylglycerols, monoacylglycerols, terpene, etc., or polar lipids like surfactant, phospholipid, free fatty acid, etc. Depending on ingredients to be delivered, neutral or polar lipids with size ranging from few nanometers to few hundred nanometers are employed. In this case, it may be stated that the strategy of surfactant micelle-based delivery is suitable for small-sized nanoparticle, while oil droplet or solid is preferred for others.

- Natural protein nanoparticles in the form of casein micelle, found in bovine milk and other dairy products, are being widely consumed by us starting from ancient time. Similar to lipid, protein nanoparticles are also used in delivery system for color, flavor, preservatives, vitamins, minerals, etc. Protein nanoparticles of different sizes ranging from few nanometers (globular protein) to several nanometers (micelle, zein, gliadin, whey protein, etc.) are prepared from protein molecules by physical interactions like hydrophobic, van der Waals, hydrogen bonding, and electrostatic interaction or covalent bonding according to requirement. Physical bond primarily depends on reaction conditions, determined by nature, pH, ionic strength of solvent, etc., while covalent bond gets formed only at a particular physicochemical condition. Generally, protein nanoparticles are prepared in spherical shape, but in some cases fibrous structure is required to develop delivery system. Fate of the protein nanoparticles in gastrointestinal tract significantly depends on their shape, size, and surface charge.
- Digestible or indigestible carbohydrate nanoparticle which is defined as assembled structure of polysaccharides has been successfully adopted to develop delivery system of functional ingredient. These are synthesized from starch, cellulose, pectin, xanthan, etc. Carbohydrate nanoparticles can be prepared either by top-down approach by breaking larger structured polysaccharides such as starch, cellulose fibril, chitosan fibril, etc., or bottom-up approach starting from polysaccharide molecules. In general the former strategy leads to formation of spherical nanoparticles, whereas the latter one gives both spherical and fibrous nanostructures. It has been studied that the digestion of the carbohydrate nanoparticles significantly depends on source from which it has been derived. For example, certain type of starch-derived carbohydrate readily gets hydrolyzed by amylases in upper gastrointestinal tract, but there also exists polysaccharide-derived carbohydrate, those that don’t get digested in upper gastrointestinal tract; rather they are fermented in lower gastrointestinal tract in the presence of enzymes.

10.3 Nanotechnology in Food Packaging

10.3.1 Freestanding Packaging

Food packaging is another essential aspect of food industry which needs care, since good packaging can only protect food from degradation retaining food quality, increasing shelf life, providing nutritional information to consumers, etc. In addition, packaging materials must have high mechanical strength, stability, temperature resistivity, etc. Earlier, various degradable copolymers, viz., ethylene vinyl alcohol, polyimides, polyethylene terephthalate, etc., or biopolymers, viz., polylactic acid, polystyrene, etc., were used as packaging materials (Mahendra 2017).

With global change in environment, these packaging materials are found to be not very suitable to protect food from bacterial contamination, global warming, O_2, CO_2-induced degradation, etc. Nanotechnology has helped to develop next-generation packaging system. Nanocomposite polymers like polymer silicate nanocomposite developed with the help of nanotechnology offer improved gas barrier property, heat resistance, etc.; potato starch and calcium carbonate-based nanocomposite system exhibits higher thermal stability and biodegradability and is likely to replace polystyrene-based packaging system, used for fast food (Moraru et al. 2003). Strategically, earlier polymer matrices, used as packaging materials, are now mixed with various organic and inorganic nanoparticles (silica, inorganic clay, organic clay, carbon nanotube, graphene, polysaccharide, chitosan, cellulose, ZnO, TiO_2, etc.) of different shape and size to prepare more reliable and versatile packaging material. As guided by nanotechnology, the ratio of largest to smallest filler material plays a crucial role in determining the efficiency of nanocomposites as packaging material. Among them, silicate nanoplate-based montmorillonite has gained attention as filler material due to formation of octahedral sheets of $Al(OH)_3$ between silica tetrahedral bilayers (Weiss et al. 2006). Also cellulose nanofibers have been identified as an attractive reinforcement material, since their incorporation into polymer matrix improves strength, moisture barrier, and thermal resistance of base matrices along with their support for the growth of other nanomaterials (Podsiadlo et al. 2005). Another study reveals that cellulose-starch nanocomposite can lead higher thermomechanical property and biodegradability in addition to reduced water sensitivity (Sharma et al. 2017). In the development of plastic or for glass bottles and cartons, impermeable to gases like CO_2, O_2, etc., nanoclay-based materials have got a lot of attention and are being readily used in the market as packaging material of meat, fruit, etc. Here, nanoclay material having ability to block gases, moisture, etc., is dispersed in plastic matrix such as Durethan. In some cases, clay-ethylene-vinyl-alcohol copolymer-polylactic acid biopolymer nanocomposite has been developed with improved oxygen barrier property, to increase shelf life of food products. Therefore, it can be stated that these newly developed packaging systems have multifunctional characteristics and can be divided into the following categories:

10.3.1.1 Improved Packaging

Nanoparticle and polymer-based nanocomposites are used as packaging material with improved gas, moisture barrier properties, as well as heat resistance ability. Packaging materials come in direct contact with food. The main strategy is to prevent food by maintaining inert and low-oxygen environment that inhibits microbial growth and spoilage. Nanomaterials, used as filler to the packaging polymer matrix, act as an impermeability barrier, and the impermeable property gets increased significantly due to larger surface area of the nanomaterials. Nanoclay which is the polymer nanocomposite with complex metallic ores, obtained from volcanic ash as montmorillonite, provides very good barrier to gas permeability. Different polymers such as nylons, polyolefin, PET, epoxy resin, and poly methane are found suitable here. As studied by Park et al., thermoplastic starch/clay nanocomposite with higher tensile strength and lower water vapor permeability in contrast to bare thermoplastic starch is being widely used in food industry (Park et al. 2003).

10.3.1.2 Active Packaging

The active packaging provides the inert barrier between the food product and outside environment and reacts with the food product for extension of the shelf life and good conditions (Grumezescu 2017). Components of the active packaging for food include (1) nanocomposites (metal ions of silver, copper, and gold and metal oxides of TiO_2 and MgO), (2) antimicrobial films (antibacterial/antifungal compounds like sodium benzoate and benomyl, acid, silicate, ethanol, zinc, elements (Si, Na, Al, S, Cl, Ca, Mg, Fe, Pd, and Ti), edible clove, pepper, cinnamon, coffee, chitosan, antimicrobial lysozyme, and bacteriophages), and (3) gas scavengers [TiO_2, iron powder, silicates, sulfites, chlorides, polymeric scavengers, elements (Fe, Si, Ca, Al, Na, Cl, K, Mg, S, Mn, Ti, Co, V, Cr, and P)] (Ahvenainen 2003; Brockgreitens and Abbas 2016). Enzymatic oxygen scavenging is achieved by the chemical reaction between alcohols generated in the food product and the alcohol oxidase enzyme**.**

Nanoclay-polymer composites were used for improving of gas barrier properties of food packages. The most often used one is montmorillonite (also called as bentonite), with natural nanoscalar layered structure that can restrict the permeation of gases when incorporated into a polymer.

Here, polymer and inorganic material (Ag, TiO_2, MgO, CuO, etc.) nanocomposites are used in packaging system. Ag nanoparticles have potency of antimicrobial activity and anti-odorant efficiency and are being widely used as health supplements and food packaging. Due to biocompatibility, SiO_2 nanoparticles are used in direct food contact applications, packaging, in the clearing of beers and wines. Nanosized TiO_2 are often incorporated into the packaging materials for photocatalytic sterilization. Other important achievements of nanotechnology are the incorporation of efficient nanosensor into the packaging system to detect food spoilage due to oxygen and moisture (Lagarón et al. 2005). Nanocomposite of oxide nanoparticles and food-grade polypeptide is being widely used as oil packaging system that protects oil from oxidation due to their antioxidant property (Scheffler et al. 2010). Composite of silica gels and thiosulfate is being used as packaging material due to their long-lasting antibacterial activity (Gupta and Silver 1998). Silver zeolite, made from alkaline earth metal, aluminosilicate, followed by partial replacement by silver ions, shows antimicrobial activity in nano-form, ascribed to reactive oxygen species (ROS) generation. Silver zeolite is being used to various products, viz., food preservation, disinfections of medical products, etc., as antimicrobial coating of food (Kawahara et al. 2000; Matsumura et al. 2003). In this context, different noble metal and metal oxide-based antimicrobial materials, carbon nanotubes having improved mechanical barrier, have been developed as waxy nano-coating materials to extend shelf life of foods. Among various antimicrobial nanomaterial agents, TiO_2 and Ag have gained attraction as they highly generate ROS preventing foodborne pathogens and food spoilage. For example, Ag/TiO_2 and Ag/carbon nanotube nanocomposites show significantly enhanced antimicrobial activity against *E. coli* and *Bacillus cereus* spores exhibiting their importance as packaging materials (Krishna et al. 2005). Ag-doped TiO_2 nanoparticles exhibit antimicrobial activity against *Bacillus cereus* spores on aluminum and polyester coating surfaces and also destroy several airborne bacteria (Vohra et al. 2006). Sodium dodecyl sulfate (SDS) and polyvinylpyrrolidone (PVP)-stabilized Ag nanoparticles show enhanced antimicrobial activity against *E. coli* and *Staphylococcus aureus*.

10.3.1.3 Smart Packaging

Smart packaging can be defined as small and inexpensive labels or tags attached onto primary packaging such as pouches, trays, and bottles or more often onto secondary packaging such as shipping containers to facilitate communication throughout the supply chain (Yam et al. 2005). Carbon dioxide absorbers and generators can be used in sachet or label form to reduce the respiration rate, and eliminate or minimize the oxygen concentration, which can cause quality deterioration of fruits and vegetables. Modified atmospheric packaging (MAP) is applied to extend the shelf life by removing or replacing the air surrounding food products, altering the amounts of carbon dioxide (CO_2) and nitrogen (N_2), and eliminating extension of the postharvest life, thus prolonging the primary fresh conditions of food products (vegetables, fruits, meat, and fish). However, use of some components in the food packages and preservative manufacture such as silver, surfactant, lipid, protein, carbohydrate nanoparticles, and carbon nanotubes in emulsified form caused health impairments.

These types of packaging systems include sensor to detect any internal or external biochemical and microbial changes in food product and packaging materials, thus helping to take necessary initiative in food security. For example, nanosensor, used in plastic packaging system, changes its color in the presence of gases in food when it gets spoiled. Several types of nanomaterial-based sensor, electronic nose, biosensor, and nano-test strip have been developed by researchers for smart packaging. For example, Mills et al. have developed nanosensor where oxygen gets employed as an indicator to monitor uncooked bacon under CO_2 environment (Mill 2005). Nanosensor based on carbon nanotube functionalized with *Salmonella* antibody is used to detect pathogenic infection on the surface of food such as chicken (Villamizar et al. 2008)). Chemical sensors, developed on the basis of xanthine and hypoxanthine, are being successfully used to monitor freshness of canned tuna (Çubukçua et al. 2007). Companies like Nestle, Monoprix supermarket, and British Airways are already using colorimetric chemical sensors to identify food spoilage that significantly improves food transportation (Pehanich 2006).

10.3.1.4 Intelligent and Responsive Packaging

Intelligent packaging is capable of carrying out detecting, sensing, recording, tracing, communicating, and applying scientific logic to extend shelf life, enhance safety, improve quality, provide information, and warn about possible problems (Yam 2009). The components of the intelligent packages are (1) indicators (methylene blue, acid, antioxidants, mineral oil and sugar, elements (Na, K, Ca, Si, Al, and Mg). Responsive packaging technologies are based on the integration of a sensor or sensing interface on the packaging film in real-time and continuous quality monitoring by implementing three important design features: prevention of cross-contamination, selection of the target analytes, and choice of the transduction system. The components of responsive packages are stimuli-responsive materials, hydrogels, surfaces, particles, and supramolecules (Brockgreitens and Abbas 2016). Oxygen scavengers used in the intelligent packaging prevent microbial growth, off flavors and off odors development, color changes and nutritional losses, and reduction in the shelf life of foods based on the following mechanism of iron oxidation.

10.3.2 Nanolaminates

Nanolaminate which consists of chemically or physically bounded few layers of nanomaterials like proteins, polysaccharides, lipid, etc., is the new strategic concept that has been added to packaging system as edible packaging material for fruit, meats, clothes, chocolate, candies, etc. Due to its extreme low thickness, it is better to be coated on food materials, rather than use as freestanding packaging materials. Nanolaminates also offer higher moisture, gas, and lipid barrier along with textural properties (Morillon Vet al. 2002; Phan et al. 2008). Continuous use of earlier non-degradable packaging materials is contaminating our environment and becoming a main threat globally. In contrast, nanolaminates have been developed from biodegradable nanoparticles like proteins, carbohydrates, and lipids, obtained from plant and renewable resources. But these natural nanoparticle layers have their own advantage and disadvantage. For example, lipid layer has high barrier to moisture, but suffers from mechanical strength and other gas impermeability. On the other hand, polysaccharide and protein nanoparticles are effective to gas barrier, but not to moisture. In this context, nanotechnology has helped to develop efficient nanolaminating material out of these. For example, cellulose nanoparticle, obtained from corn, when added with nanoclay exhibits excellent mechanical, stability, and gas barrier property suitable for nanolaminates.

10.4 Types of Nanoparticles Applied in Food Systems

10.4.1 Inorganic Nanoparticles

Many types of nanoparticles used in foods are mainly composed of inorganic materials, such as silver, iron oxide, titanium dioxide, silicon dioxide, or zinc oxide. These particles are either crystalline or amorphous solids at ambient temperature, which may be spherical or non-spherical, have different surface characteristics, and come in different sizes depending on the initial materials and preparation conditions used in their fabrication. Inorganic nanoparticles also vary in their tendency to dissolve under different solution conditions (such as pH and ionic strength) and in their chemical reactivities, which has a major impact on their GIT fate and toxicity.

10.4.2 Organic Nanoparticles

This type of nanoparticle is primarily composed of organic substances, such as lipids, proteins, and/or carbohydrates. These substances tend to be liquid, semisolid (gelled), or solid (crystalline or amorphous) at ambient temperatures depending on their composition and processing conditions. Most organic nanoparticles commonly used in foods are spherical, but they may be non-spherical under some circumstances (e.g., nanofibers). Organic materials vary considerably in their behaviors within different regions of the human GIT, e.g., they may dissolve, precipitate, aggregate, or be digested in the mouth, stomach, small intestine, or colon depending on their compositions and structures. In general, it is thought that organic nanoparticles are less toxic than inorganic ones, because they are often fully digested within the human GIT and are not bio-persistent. Nevertheless, there may be certain circumstances where they could cause toxicity.

10.5 Types of Nanoformulations

10.5.1 Nanoencapsulation

Nanoencapsulation in food processing is defined as technology to pack substances in miniature at the nanoscale and also improve food rehydration; edible coatings are used on fruits, vegetables, meats, chocolate, candies, and bakery products, by serving as moisture, lipid, and gas barriers. Nanoencapsulation is applied for manufacturing of confectionary products, pharmacological and dietary supplements, and nanocarrier food systems (Ezhilarasi et al. 2013). Nanoencapsulation can be achieved by using three methodologies as explained in Fig. 10.3: (1) physical processes based on spray drying-coating, extrusion, and spray drying, (2)

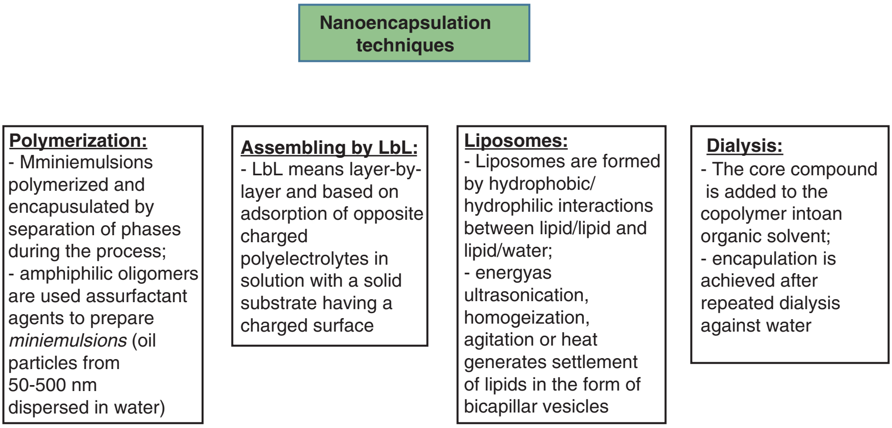


Fig. 10.3 Nanoencapsulation techniques

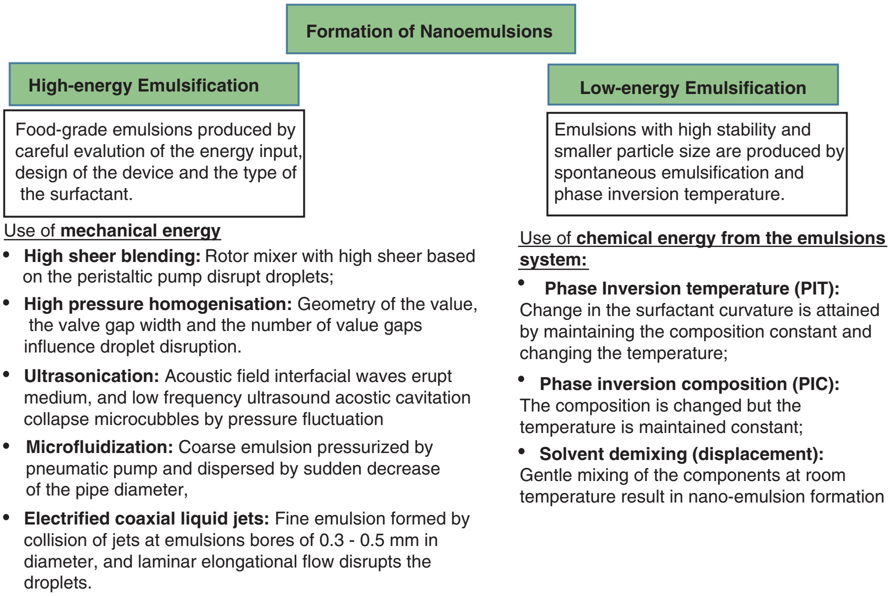
physiochemical processes based on the simple or complex coacervation and entrapment into liposomes, and (3) chemical processes based on interfacial polymerization and molecular inclusion (Quintanilla-Carvajal et al. 2010).

Three types of nanosystems as wall materials used in nanoencapsulation of food ingredients are as follows: (1) lipid-based nanosystems including nanoliposomes, nanocochleates, colloidosomes, solid lipid NPs, and archeosomes; (2) polymeric-type nanosystems including nanofibers, carbohydrate-based NPs, starch, cellulose, pectin, guar gum, alginate, chitosan, and dextran; and (3) protein-based nanosystems including corn protein or zein ultrafine fibers and milk protein nanotubes.

10.5.2 Nanoemulsification

Nanoemulsification in food processing is preparation of nanoemulsions by using techniques specified in Fig. 10.4, such as “phase inversion composition, high-pressure homogenization, ultrasonication and microfluidization” (Quintanilla-Carvajal et al. 2010). Nanoscale delivery systems such as microemulsions, emulsions, nanoemulsions, multiple emulsions, multilayer emulsions, solid lipid nanoparticles, liposomes, biopolymer nanoparticles, and microgels need to be selected for particular application in food industry based on the following criteria: (1) safety, (2) commercial viability and food matrix compatibility, (4) robustness, (5) performance, and (6) labeling requirements (McClements et al. 2015).

Hydrocolloid proteins derived from milk, egg, soy, collagen, and gelatin are most commonly used as emulsifiers in food emulsions.



Food-grade emulsions produced by careful evalution of the energy input, design of the device and the type of the surfactant.

Use of **mechanical energy**

- **High sheer blending:** Rotor mixer with high sheer based on the peristaltic pump disrupt droplets;
- **High pressure homogenisation:** Geometry of the value, the valve gap width and the number of value gaps influence droplet disruption.
- **Ultrasonication:** Acoustic field interfacial waves erupt medium, and low frequency ultrasound acostic cavitation collapse microcubbles by pressure fluctuation
- **Microfluidization:** Coarse emulsion pressurized by pneumatic pump and dispersed by sudden decrease of the pipe diameter,
- **Electrified coaxial liquid jets:** Fine emulsion formed by collision of jets at emulsions bores of 0.3 - 0.5 mm in diameter, and laminar elongational flow disrupts the droplets.

Emulsions with high stability and smaller particle size are produced by spontaneous emulsification and phase inversion temperature.

Use of **chemical energy from the emulsions system:**

- **Phase Inversion temperature (PIT):** Change in the surfactant curvature is attained by maintaining the composition constant and changing the temperature;
- **Phase inversion composition (PIC):** The composition is changed but the temperature is maintained constant;
- **Solvent demixing (displacement):** Gentle mixing of the components at room temperature result in nano-emulsion formation

Fig. 10.4 Methods of formation of nanoemulsions

10.6 Characteristic Parameters of Nanoparticles for Application in Food Systems

The nanoparticles found in food and beverage products vary considerably in their physiochemical and structural properties, which determines their GIT fate and propensity to cause toxicity (Fig. 10.5).

- Based on their composition, the nanoparticles found in foods may consist of inorganic (e.g., silver, titanium dioxide, silicon dioxide, iron oxide, and zinc oxide) and/or organic components (e.g., lipids, proteins, and carbohydrates).
- The ability of inorganic nanoparticles to produce toxicity is often associated with their chemical reactivity, which depends on their composition. For example, some inorganic nanoparticles dissolve and release ions that promote undesirable chemical or biochemical reactions (e.g., silver nanoparticles), whereas others are relatively inert (e.g., titanium dioxide nanoparticles).
- Dimensionally, food nanoparticles vary considerably in their dimensions, ranging from a few nanometers (surfactant micelles) to a few hundred nanometers (lipid, protein, or carbohydrate nanoparticles), depending on the materials and processes used to create them.
- Nanoparticle dimensions influence their GIT fate and toxicity through a number of mechanisms. Smaller nanoparticles are usually dissolved or digested more

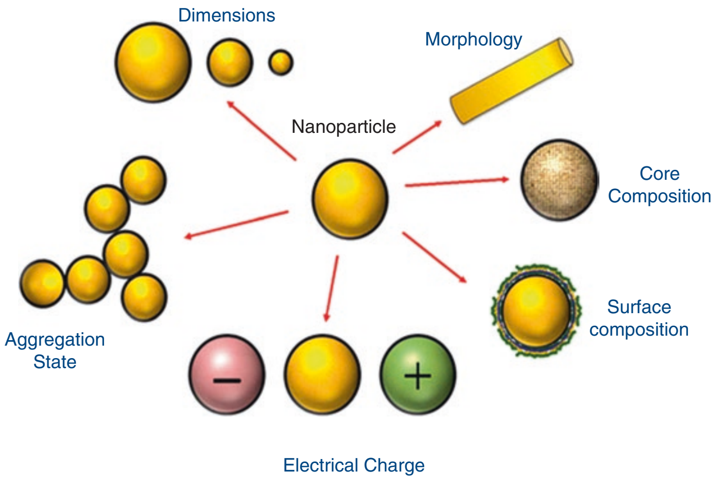


Fig. 10.5 Characteristics of nanoparticles

rapidly in GIT fluids and have higher interacting ability with GIT components (such as digestive enzymes, phospholipids, bile salts, or mineral ions), and consequently their penetration through the mucus layer coating epithelium cells usually increases as their size decreases.

- Interfacial properties of nanoparticles in foods and within the GIT are influenced typically by a surrounding coating of adsorbed substances (sometimes referred to as a "corona"), which determines the electrical charge, hydrophobicity, thickness, digestibility, and chemical reactivity of the interface. These surface properties will determine the behavior of the nanoparticles in the GIT.
- Food-grade nanoparticles may exist as isolated individual particles, or they may form clusters that vary in size, morphology, and strength. Typically, nanoparticles in clusters are held together by physical forces, such as Van der Waals, electrostatic, hydrogen bonding, and hydrophobic forces. The aggregation state of the nanoparticles is therefore often highly dependent on environmental conditions, such as pH, ionic strength, ingredient interactions, and mechanical forces. The dimensions of nanoparticle clusters may be much greater than the dimensions of the individual nanoparticles, which has a major impact on their GIT fate, such as their ability to move through the gastrointestinal fluids, mucus layer, or epithelium cells.
- A major factor that has been frequently ignored in the studies of the biological fate of ingested food nanoparticles is their interactions with various components within complex food matrices and GIT.
- Prior to ingestion, nanoparticles are typically dispersed within food matrices that vary considerably in their compositions, structures, and properties. Foods are assembled into different structural features (such as bulk phases, biological cells, polymers, droplets, bubbles, particles, and networks), and they are produced using various processing operations (such as mixing/separation, cooling/heating,

concentration/dilution, hydration/dehydration, and mechanical action). The physicochemical and structural properties of nanoparticles may therefore be changed considerably when they are dispersed in food products, which would play an important role in determining their subsequent GIT fate and toxicity.

- The interaction between these food components and nanoparticles may significantly alter the biological fate of these nanoparticles.

10.7 Synthesis of Nanomaterials

Nanoparticles, used in different section of the food chain, vary significantly in their physicochemical and structural properties determining their efficiencies in food system starting from processing to packaging and their fate in gastrointestinal tract. The variation of physicochemical and structural properties of the nanoparticles considerably depends on the shape and size of the nanomaterials, those that get tuned by their synthesis methods. Hence, for multipurpose uses of nanomaterials in food system, nanomaterials with suitable shape, size composition, and interfacial properties have to be designed properly, and experimentally it is only achieved by different synthesis processes. In general, nanomaterials, used in various sections of the food chain, can be divided into two categories, inorganic and organic, and primarily this is the factor on which fate of the nanoparticles in gastrointestinal tract depends.

Inorganic nanomaterials, viz., Ag, TiO_2, SiO_2, Fe_2O_3, ZnO, etc., either in amorphous or crystalline form, are already reported to be used in the food chain regularly, starting from active ingredient to gas-impermeable barrier to smart packaging materials having nanosensor. They are present in food system either in spherical or in non-spherical shape and different dimensions ranging from few nanometers to several nanometers. These nanoparticles are metal oxide (TiO_2, SiO_2, Fe_2O_3, ZnO, MgO, etc.) nanoparticles and noble metal, viz., Ag, nanoparticles.

10.7.1 Methods for Synthesis of Metal Oxide Nanoparticles

Among various metal oxide nanoparticles, used so far in the food chain, TiO_2 nanoparticles have gained much attention as functional ingredient of certain food product to increase their lightness and brightness. TiO_2 nanoparticles (<100 nm) are being widely used in chewing gum, candies, dessert, beverages, etc., as food colorant as well as UV-resistant food packaging material, and anatase phase is preferred in food application because of its less toxicity. Spherical and amorphous SiO_2 nanoparticles having size of 100–1000 nm (food grade: E551) are used in powdered foods as anticaking agents to increase flow properties including salt, spices, dried milk, icing sugar, etc. ZnO nanoparticles are being used as sources of Zn, in supplements and functional foods, since Zn is highly essential to maintain food and well-being. In food packaging Zn nanoparticles are used due to their antibacterial activity and UV absorption property. However, a recent study illustrates that high intake of ZnO nanoparticle causes adverse effect to human, and intake depends on the size of nanoparticles. Fe_2O_3 nanoparticles are significantly utilized as colorant and source of bioavailable Fe. Few of the synthesis strategies of metal oxide nanoparticles are briefly described below.

10.7.1.1 Coprecipitation Method

Coprecipitation is one of the most adopted strategies to synthesis of metal oxide nanoparticles since monodisperse particle could be easily prepared by this method along with scalability. The synthesis method involves coprecipitation of solids from aqueous/nonaqueous solutions followed by thermal decomposition of the precipitate. Nucleation and growth determining size distribution of the particles get tuned by reaction parameters such as precursors, sequence of reaction, solvent, etc. Slow-growth mode follows Oswald ripening process giving uniform size, while fast-growth mode generates nonuniform size and irregular shape. As the system, overall, would try to minimize energy, thus smaller-sized particle would diffuse into the solution and get adsorbed on the surface of the bigger-sized particles. Nanoparticles of ZnO, TiO_2, and Fe_2O_3, used in the food chain, have been synthesized by this process from respective chloride salt. Often, precipitate exists in amorphous form; thus subsequent heating or annealing is necessary for rendering crystallinity into them. As an example, TiO_2 nanoparticles (~40–100 nm) have been synthesized in aqueous medium by precipitating $TiCl_3$ in the presence of NH_4OH (Borse et al. 2002). Though aqueous medium is cost-effective, truly monodisperse nanoparticle can’t be prepared by this technique. In contrast, nonaqueous medium offers higher control over size of the nanoparticles. However, nonaqueous medium requires significantly higher time (from several days to week) and involves more toxic solvents, but it is preferred in some cases for monodisperse size. For example, monodisperse Fe_3O_4 nanoparticles (~4 nm) have been prepared by mixing $Fe(acac)_3$ with oleylamine and diphenyl ether (Sun and Zeng 2002). Fe_2O_3 nanoparticles of this size range are very suitable for their utilization as bioavailable source of Fe, since Fe easily gets leached out from surfaces of these small-sized nanoparticles. Coprecipitation technique generally produces spherical nanoparticles, but anisotropic nanoparticle can also be synthesized in the presence of surfactant.

10.7.1.2 Sol-Gel Method

Sol-gel technique was adopted to synthesize metal oxide nanoparticles. The process consists of hydrolysis followed by condensation of a liquid precursor that leads to formation of solid-phase network finally giving nanoparticles. It can be divided into two distinct steps, sol and gel formation. Sol consists of stable colloid suspension of precursor in liquid phase or stable solution. When sol turns into a network structured porous semisolid diphasic system, it is termed as gel. Gel is then dried at elevated temperature to remove liquid for densification, followed by thermal decomposition or annealing to achieve crystalline nanoparticle. Hydrolysis and condensation affect the formation of sol and gel and consequently affect the size and composition of the nanoparticles. Hydrolysis and condensation, hence sol-gel formations, are controlled by reaction parameters such as precursor, solvent, pH, temperature, etc. Finding of suitable precursor and solvent (aqueous/nonaqueous) is the main key of this strategy to synthesize monodisperse nanoparticles. As an example, four-coordinated tetraethoxy [$M(OEt)_4$, M = Si, Ti, Zr] compound is generally preferred for the preparation of metal oxide nanoparticles. Partial charge on metal ion generally causes hydrolysis which subsequently controls the shape and size. Higher partial charge causes higher reaction rate preparing nanoparticles of irregular shape and size. To decrease reaction rate, often transitional metal ions are dispersed in the aqueous solution. Here, water molecules react with transitional metal ions by transferring electrons to empty "d" orbital of the transition metal ions forming $[M(OH)_x]^{Z+}$, where x and Z represent coordination number and charge of the transition metal ions; thus hydrolysis kinetics yielding $[M = O]^{(Z-2)+}$ gets to slow down. Sometimes, hydrolysis kinetic is also tuned by controlling the amount of water (in case of aqueous reaction) and organic solvent (in case of nonaqueous reaction). A larger variety of metal oxide including TiO_2, ZnO, MgO, CuO, Cu_2O, and SiO_2 nanoparticles having a wide range in size has been synthesized by this method using a wide variety of precursors like halides, acetylacetonates, alkoxides, etc. As an example, the following reaction has been assigned for the growth of TiO_2 using alkoxide as precursor (Andhariya et al. 2013):

$$\mathrm{Ti(OR)_3 + H_2O \rightarrow Ti(OH)_3 + R_2O}$$
$$\mathrm{Ti(OH)_3 \rightarrow TiO_2 + H_2O}$$

Here, "R" represents the alkoxide group. Apart from metal oxide with antimicrobial activity, sol-gel processing is also widely used to prepare SiO_2 nanoparticle which is used as carrier of functional ingredient in food system. Tetramethoxysilane (TMOS) and tetraethoxysilanes (TEOS) are mostly used as precursors for this purpose. When water is added to TMOS or TEOS, OR functional group of TMOS or TEOS is hydrolyzed by OH^- ions that can be described by the following reaction:

$$\mathrm{Si(OR)_4 + H_2O \rightarrow HO\text{-}Si(OR)_3 + R\text{-}OH}$$

After hydrolysis, condensation takes place according to the following reaction which subsequently reduces three-dimensional Si-O-Si network structure:

$$\mathrm{2HO\text{-}Si(OR)_3 \rightarrow (OR)_3\,Si\text{-}O\text{-}Si(OR)_3 + H_2O}$$

or

$$\mathrm{2OR\text{-}Si(OR)_3 + 2OH\text{-}Si(OR)_3 \rightarrow (OR)_3\,Si - O - Si(OR)_3 + R\text{-}OH}$$

The main disadvantage of the current strategy is the van der Waals force-induced aggregation which enhances size of the nanoparticles decreasing their efficiency as functional ingredient carrier. Increasing surface charge or incorporating steric hindrance by functionalization by reactive ligands is adopted to prevent aggregation. Depending on removal process of solvent, sol-gel strategy could be classified into "aerogel" and "xerogel" processes. When pore fluid of gel is removed in gaseous phase, a very low-density network forms at some hypercritical condition, and then it is defined as "aerogel." The characteristic feature of aerogel is that its shape remains unchanged and this process is very useful for the synthesis of assembled structures. When pore fluid is getting removed at ambient pressure at elevated temperature, network gets shrunk further into dense gel, and then it is called xerogels. Drying stress destroys monolithic gel bodies giving powder. Primary advantages of this technique are the versatility and flexible rheology that allows easy shaping and embedding. This method provides a significant homogeneity for multicomponent system, particularly suitable for laminating purpose.

10.7.1.3 Microemulsion Method

Here, a molecule with both hydrophilic and hydrophobic moiety, defined as amphiphilic surfactant molecule, is used as basis of this process. Due to this dual character, amphiphilic molecules get accumulated at water-air interface to reduce surface tension, and such accumulation results in a self-assembled order in bulk solution. In a mixture of organic solvent and water, amphiphilic molecules accumulate at their interface. In aqueous solution, hydrophobic tail of the surfactant molecule avoids contact with water and as surface concentration crosses a critical value, micelle gets formed. Micelles have the dimension ranging from few nanometers to micrometers depending on water, solvent ratio, and surfactant concentration. These micelles act as a reactor for the growth of metal oxide nanoparticles including ZnO, TiO_2, SiO_2, Fe_2O_3, etc. Addition of co-surfactant gives anisotropic micelle which results in non-spherical structure. Notably, unlike conventional emulsion technique, no shear condition is required for microemulsions. Depending on hydrophilic head, surfactants are divided into ionic and nonionic surfactant. There also exists another type of surfactant, named as zwitterionic surfactant, which contains both anionic and cationic hydrophilic head. Amphoteric surfactant has gained attention due to its ability to synthesize a wide varieties of nanostructures. This ability may be attributed to the change of its hydrophilic head from cationic and zwitterionic to anionic with increasing pH of the medium. Microemulsion process is classified into two categories, direct microemulsions in which organic solvent is dispersed in water and reverse microemulsions where water is dispersed in organic solvent.

10.7.1.4 Microwave Method

Here, electromagnetic energy is being converted into heat energy that is required to carry out chemical reaction. This technique is found to be more efficient in comparison to conventional heating process, as this produces heat homogeneously. Two mechanisms, dipolar polarization and conduction, are involved here for the conversion of energy. In polarization mechanism, dipole when irradiated to microwave generates heat, attributed to collision between dipoles in the presences of rapid oscillating electric field of microwave. Frequency of the microwave plays a crucial role for heat generation. There exists an optimum frequency which depends on the nature of solvent at which generation of heat is maximum. Conduction mechanism occurs when solvents have free charge carrion in the form of either electron or ion. Here, heat is being generated due to conventional Joule heating mechanism. Microwave can easily penetrate the material; thus heat is being generated throughout the volume of the system, defined as volumetric heating, which results in high reaction rate, smaller particle size, narrow distribution in particle size, high purity, enhanced physicochemical properties, etc. Temperature, generated in this process, significantly depends on the dipole moment of the solvent; the higher the dipole moment, the higher the temperature. With the advancement of microwave technology, it is possible to control synthesis parameters; thus monodisperse particles are easily being generated. Various metal oxide nanoparticles and structures, used in food industry, like ZnO, TiO_2, CuO, and MgO, have already been synthesized by this technique. For example, Komarneni et al. synthesized TiO_2 microsphere by this technique (Komarneni and Roy 1985).

10.7.1.5 Chemical and Reduction Method

Chemical reduction is largely utilized to synthesize noble metal, i.e., Ag nanoparticles, as size could be easily controlled by this method. Here, $AgNO_3$ salt is mostly used as precursor, while borohydride, citrate, elemental hydrogen, ethylene glycol, glucose, etc., are generally used as reducing agent. The main problem of this process is the agglomeration, but it has been taken care of by stabilizing agents like polyvinylpyrrolidone (PVP), oleylamine, sodium dodecyl sulfate, N,N-dimethylformamide (DMF), etc. Concentration of the reducing and stabilizing agents plays a crucial role to determine size of the nanoparticle. Later, solubility of the nuclei determines the stage at which nucleation would be stopped and agglomeration to take place.

10.7.1.6 Sonochemical Method

Sonochemical method utilizes ultrasound to cause chemical reaction and basic principle can be described as follows: when liquids is exposed to intense ultrasound, acoustic cavitation which is the combination of formation, growth, and implosive collapses of bubbles occurs and leads to chemical reaction. Ultrasonicator which consists of piezoelectric transducer is used to convert conventional electrical supply into mechanical vibrational energy (~100 W/cm^2, 20 kHz–1 MHz). When vibration is allowed to pass through liquid, liquid medium alternatively gets compressed and relaxed at this frequency resulting in formation of microscopic bubbles. These bubbles implode during compression and expand during decompression. During this oscillatory compression and expansion, bubbles grow, and after reaching a maximum size, they collapse that generates high pressure and temperature. When large number of such events occurs successively within few microseconds, high energy is being generated in the medium due to cumulative effect. In sonochemical process, this energy is utilized for chemical reduction, particularly for reduction of noble metals such as Ag. Briefly, OH^- ions, generated by ultrasound irradiation, reduce Ag^+ ions into Ag^0 which successively leads to formation of Ag nanoparticles. As an example, Gutierrez et al. reduced Ag^+ in aqueous solution using 1 MHz ultrasound in the year 1987. Later in 1992, Nagata et al. prepared Ag nanoparticles after reducing Ag^+ ions under Ar atmosphere by 200 kHz ultrasonicator (Nagata et al. 1992). Concentration of precursor, i.e., $AgNO_3$, plays a crucial role on the size of the nanoparticles, lower the concentration less the particle size. There is also some report on the synthesis of Ag nanoparticles in alcoholic medium. Alcoholic medium offers faster reaction kinetics due to enhanced generation of OH^-. It has been studied that length of alkyl group affects significantly on the size of the nanoparticle (Caruso et al. 2002). Surfactants and organic additives such as polyethylene glycol, sodium dodecyl sulfate, etc., are often used to control size and stabilize the nanoparticles (Mizukoshi et al. 2001). Recently, sonochemical synthesis has been tried out in different gaseous environments in order to investigate their effect on reduction, and the study illustrates the reduction in the following order $N_2 = He < Ne < Ar < Kr$ (Okitsu et al. 2002). The observed phenomena can be explained as follows: the higher the ratio of C_p/C_v (specific heat ratios), the lower the thermal conductivity of the purging gas within the liquid and then the higher the temperature of collapsing bubbles that results in higher reduction rate. In addition, higher solubility of the gas also enhances reaction rate. Temperature is another factor affecting reduction reaction. Initially rate increases with temperature and finally it decreases, i.e., there exists an optimum temperature to carry out the reduction process using this strategy. In general, reduction rate gets decreased with increasing ultrasound frequency. One of the major advantages of this process is that bimetallic nanoparticles can also be synthesized those that often exhibit advantage over single-phase nanoparticles. In addition to colloidal particles, thin film of Ag nanoparticle can also be synthesized by this technique. As an example, Ag nanoparticle thin film on glass slides has been prepared using water-ethylene glycol (10 volume %) solution and $AgNO_3$ (0.05 M) solution at ambient condition using ultrasonicator of 20 kHz of frequency and 600 W/cm^2 power (Perkas et al. 2008).

10.7.1.7 Radiolysis Method

Radiolytic reduction is another method to synthesize Ag nanoparticles where Ag^+ ions get reduced by ions and radicals instead of oxygen. Hydrated ions or radicals get produced by radiations (x-ray, γ-ray, UV ray) and electron beam in aqueous medium, and the reduction reaction can be written as follows:

$$\begin{gathered} H_2O \text{ or} \\ Ag^+ + \rightarrow Ag^0 \\ Ag^+ + \rightarrow Ag^0 + H^+ \end{gathered}$$

Ag^0 acts as nucleation center and coalescence further forming nanoparticles. Sometimes dimers of Ag atoms also get formed ($Ag^0 + Ag^0 \rightarrow Ag_{2,}$ $Ag^0 + Ag^+ \rightarrow Ag_2^+$) and contribute to the formation of Ag nanoparticles. Here size of nanoparticle is controlled by concentration of precursor ($AgNO_3$) and dose of radiation. Like other processes, this also suffers from agglomeration. To prevent agglomeration, electrostatic stabilizing agents such as $–NH_2$, –COOH, –OH, etc., are used. As they have high electron affinity, they easily form covalent bond with Ag nanoparticles; thus surface activity gets reduced. Recently, polyvinyl alcohol (PVA) that prevents agglomeration by steric hindrance method is being used. –OH group, present in PVA, is strongly capable to absorb Ag^+ by forming secondary bond; thus steric entrapment occurs and agglomeration gets reduced (Gautam et al. 2006). The reaction between PVA and Ag^+ could be written as $R\text{-}OH + Ag^+ \rightarrow R\text{-}O\text{-}Ag + H^+$; here R-OH represents PVA monomer. For example, Ag nanoparticles ~7 nm have been synthesized using $AgNO_3$ (concentration $\sim 2\times 10^{-4}$ M) as precursor in alcoholic medium (2 propanol) and PVA [59]. In this context, it is to mention that PVA can also reduce Ag^+ ions into Ag^0 in the absence of any radical scavenger. Using this strategy, different nanostructure has also been achieved. For example, Wang et al. investigated that dendritic structure of Ag gets isotropic when they synthesized Ag in the presence of ethanol and $C_{12}H_{25}NaSO_4$ (Wang et al. 2000). Main advantage of this technique is that no side reaction is involved here.

10.7.1.8 Template and Hydrothermal-Assisted Method

One of the significant achievements of nanoscience and related technology is the synthesis of desire nanostructures in order to achieve required activity. It has been investigated that one-dimensional nanostructures like nanorod, nanowire, nanotube, and nanobelt exhibit better application opportunity due to their anisotropic structure rather than their isotropic spherical structure. One-dimensional structure is particularly very useful in sensor application in food monitoring. Template-assisted method is very much reliable to prepare these one-dimensional structures, and the size of the nanostructure is completely determined by pore size of the template. Templates are used as structural framework to grow nanostructures within its spatially confined space, followed by removal by calcination or chemical etching. Templates are divided into two categories: soft template such as peptide, lipid, polymer, liquid crystal, block copolymer, micelle, etc., and hard template such as anodic aluminum oxide (AAO), polycarbonate membrane, silica sphere, polymer latex, etc. As an example, ordered structure with Ag hollow sphere has been synthesized using AAO template (Chen et al. 2004). Though hard templates are robust, strong acid/base/high temperature is necessary to remove the template that diminishes the quality of the nanostructure (Lou et al. 2008). Compared with hard template, soft templates are easy to handle, making them more versatile. As an example, Zhang et al. synthesized hollow Ag sphere using PEO-b-PMAA-SDS complex micelle as soft template (Zhang et al. 2002). Spherical Ag nanoparticles with diameter ~11 nm have been prepared using poly(hydroxyethyl methacrylate) template (Salsamendi et al. 2013). Network structure consisting of cubic-shaped Ag nanoparticles has been synthesized by Chen et al. using PVP as template and ethanolic $AgNO_3$ solution (Chen et al. 2011). Recently few studies reveal that DNA can also be used as template to synthesize Ag nanoparticles (Nithyaja et al. 2012).

Template-assisted methods have limitation as few types of nanostructure are synthesized by these strategies. In contrast to template-assisted method, hydrothermal method offers to synthesize a large variety of nanostructures. Though this process was introduced by Sir Roderick Murchison more than 100 years ago, it has gained attention recently to prepare various nanostructures. Advantages of this technique are the ability to synthesize single crystalline nanostructure, nanocomposites, etc. Mechanistically, hydrothermal method has been developed on the basis of supercritical water or supercritical fluid technology. Though this technique is largely used, its chemistry is still under debate. As an example, Ag nanowires (diameter ~53 nm and length ~6 μm) have been synthesized using 100 μl of $AgNO_3$ (0.1 M) and 100 μl of sodium citrate solution (0.1 M) at 130 °C. Here, pH plays a key role; it is studied that with increasing pH, nanowire structure gets changed into nanosphere. Bari et al. (2016) have synthesized Ag nanowire (diameter ~45–65 nm and length 200 μm) using $AgNO_3$ (0.02 M, 15 ml), $_D$+ glucose (0.12 g, 5 ml) as reducing agent, and PVP (1 g, 5 ml) as surfactant. Here, hydrothermal reaction has been carried out at 60 °C for 22 h. The same group has also synthesized this film of Ag nanowire on PET substrate using hydrothermal technique. Two-dimensional arrays of Ag nanoparticles (diameter ~17 nm) have been grown by O. Ayyad et al. by this method. Here the reaction was carried out at 170 °C for 5 h, and $AgNO_3$ (0.4 g), $NaHCO_3$ (0.12 M), and PVP (2.5 g) were used as precursors (Cao et al. 2014). Here, hydrothermal reaction is carried out at 250 °C starting from $AgNO_3$, 1,2-benzenedicarboxylate (10 mM), and sodium stearate (10 mM) (Singh and Pandey 2011).

10.7.1.9 Green Synthesis

Various biological methods, known as green synthesis methods, have been developed on the basis of various microorganisms such as algae, fungi, etc., and plant extracts like leaf, fruit, flower, root, etc., in recent times. Mechanistically, Ag^+

ions get reduced by these biologically active extracts into metallic Ag which consequently forms Ag nanoparticles. These newly developed strategies offer several advantages such as their simplicity, low cost, eco-friendliness, and fast reaction rate. Some extracts also act as capping agents, so no surfactant is required to prevent agglomeration. In these methods, size of the nanoparticle is controlled by synthesis parameters like pH, temperature, reaction time, mixing ratio, etc. In addition, green synthesized Ag nanoparticles, particularly Ag nanoparticles prepared by medicinal plant extracts, are often found to be more pharmacologically active than chemically synthesized nanoparticles due to the presence of biologically active residue, attached to the nanoparticle's surface. As an example, Ag nanoparticles (~40 nm) have been synthesized by bio-reduction of aqueous Ag^+ using culture supernatant of nonpathogenic bacteria such as *Bacillus licheniformis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, etc. (Kalishwaralal 2008; Shahverdi 2007). Lactic acid present in bacteria-treated whey of buttermilk can also be used to grow silver nanoparticles (Korbekandi 2012). In order to increase synthesis yield, sometimes green synthesis is combined with other less expensive, less hazardous methods. For example, Saifuddin et al. have developed a process to prepare Ag nanoparticles by combining culture supernatant of *B. subtilis* and microwave irradiation technique in water (Saifuddin 2009). Like bacteria, various fungi have also been adopted for the synthesis of Ag nanoparticles. For example, Ag^+ gets bio-reduced into metallic Ag by an enzymatic reductive process which involves NADH of *F. oxysporum* (Ahmad 2003). In this context, Kumar et al. have demonstrated enzymatic synthesis of Ag nanoparticles of various sizes and morphologies, using α-NADPH-dependent nitrate reductase, purified from *F. oxysporum* and phytochelatin, in vitro (Kumar 2007; Prasad 2016, 2017; Prasad et al. 2018a). In this context, nonpathogenic fungi such as *Trichoderma asperellum* and *Trichoderma reesei* are found to be most suitable and are already being commercially used in food, animal feed, pharmaceutical, paper, and textile industries. Also the procurement and maintenance of the microorganism strain is difficult. This requires special culture and isolation techniques that increase the cost. The use of plant extract for the synthesis of Ag nanoparticles overcomes these problems. During recent past, significant research works have been performed on the green synthesis of Ag nanoparticles using plant extracts such as root, stem, leaves, flower, fruit, etc (Swamy and Prasad 2012; Prasad et al. 2012; Prasad and Swamy 2013; Prasad 2014; Joshi et al. 2018; Prasad et al. 2018b). Among different plant extracts, leaf extract exhibits significant potency as major bioactive molecules like glucose, fructose, chlorophyll, etc., causing reduction of Ag^+ into metallic Ag are present there. Several groups reported the green synthesis of silver nanoparticles from silver salts using leaf extract of parsley (*Petroselinum crispum*), celery, etc. Among fruit extracts, apple and cucumber extracts show potency for the synthesis of spherical Ag nanoparticles (~10 nm). It has been studied by our research group that underground root or stem extracts are rich source of bioactive reducing molecules like amino acid, protein, thiamine, ascorbic acid, etc.; thus they readily can be used to prepare Ag nanoparticles. Infusion of potato tuber seemed to be a good choice here. Amide and amino group, present in potato infusion, also act as capping agent and thus consequently help to stabilize the nanoparticles.

10.7.2 Methods for Synthesis of Organic Nanoparticles

Organic nanoparticles are manufactured for specific purposes such as encapsulation of nutrients to improve their bioavailability or mask undesirable taste or odor, etc. In general, they exist in three different forms: (1) monomer, i.e., single molecules; (ii) oligomer, i.e., monomer linkage in short chain; and (iii) polymer, i.e., monomer linkage in long chain and comes in direct contact of bloodstream via gastrointestinal tract. Depending on synthesis and composition, organic nanoparticles may exist in different forms such as liquid, semiliquid, and solid (crystalline/amorphous), significantly impacting activity. In comparison to inorganic nanoparticles, organic nanoparticles are less toxic and often get digested within human gastrointestinal tract.

10.7.2.1 Synthesis of Chitosan

Among various organic nanoparticles, chitosan is being widely utilized in the food chain to deliver and protect hydrophobic and hydrophilic function of bioactive ingredients (vitamin, probiotics, bioactive peptides, antioxidants, etc.) by the process of encapsulation or immobilization. Chitosan which is a partially deacetylated polymer of N-acetyl glucosamine containing β-(1,4)-linked-D-glucosamine residue with active amine group is generally synthesized from alkaline deacetylation of chitin monomer. A wide variety in application and functionality of chitosan is attributed to the presence of amine and –OH group. Chitosan, being very much bio-friendly, is being synthesized by various methods such as emulsion, ionic gelation, reverse micelle, self-assembly, etc. Among the methods, ionic gelation and reverse micelle are briefly discussed here, since these two methods only produce monodisperse chitosan nanoparticles over a wide range.

Chitosan nanoparticles are synthesized on the basis of interaction of two oppositely charged molecules. Nontoxic and multivalent tripolyphosphate, which helps gel formation by modifying ionic interaction, is generally utilized to prepare chitosan nanoparticles. In this context, it may be stated that reaction parameters such as pH, concentration, ratios of components, and mixing procedure considerably affect the size of the chitosan nanoparticles (Nasti et al. 2009). Multifunctional chitosan-Fe_2O_3 nanocomposite (50–100 nm), used as bioactive Fe source, has been synthesized by this ionic gelation method. It is achieved by cross-linking tripolyphosphate precipitate with NaOH, followed by oxidation by O_2 in HCl medium (Wu et al. 2009).

In reverse micellar process, a surfactant is dissolved in organic solvent to prepare reverse micelle, and then aqueous solution containing chitosan monomer is added to it with constant stirring. Here, the amount of the water plays a crucial role in determining the size of chitosan nanoparticles; more water gives larger-sized particles (Brunel et al. 2008). Reverse micelle strategy is found to be very much effective to prepare functionalized chitosan nanoparticles, viz., bovine serum albumin functionalized chitosan.

In addition to chitosan, other organic nanoparticles are synthesized by reprecipitation technique in which dilute solution of precursor in water-soluble media is directly injected into vigorously stirred water. Due to the sudden change of solubility of the precursor, the solute gets precipitated, forming a nanocrystal. As an example, nanoparticles of 1,3-diphenyl-5-(2-anthryl)-2-pyrazoline, used in the food chain in delivery system, are synthesized after injecting them from acetonitrile stock solution to water (Xiao et al. 2003). When acetonitrile is mixed with water, the dispersion property of the solute changes rapidly, leading to nucleation and growth of the nanoparticles. Here, size of the nanoparticles gets tuned by ripening process by regulating aging time. In this context, it may be stated that the nucleation of the nanoparticles gets initiated in the early-stage heterogeneous environment; thus broad distribution in size and shape is obtained. Recently, strategies have been developed that create homogeneous environment; thus size and shape control becomes easier. For example, perylene nanoparticles have been synthesized by reducing perylene perchlorate using bromine anions in acetonitrile solution. Here, size of the nanoparticles gets controlled by monomer concentration.

10.7.2.2 Synthesis of Polymer Fiber by Electrospinning and Electrospraying

Electrospinning and electrospraying are the two recently developed facile, cost-effective, and flexible techniques by which either fiber or structures ranging from few nanometers to several micrometers of any kind of polymer can be synthesized. Technically, the method has been developed which utilizes electrically charged jet of polymer solution to prepare fiber or particles, used for encapsulation, enzyme immobilization, food coating, laminating, etc. Mechanistically, in electrospinning or electrospraying processes, a polymer solution is spun or sprayed in the presence of high electric field to obtain a desired fiber or particle, respectively. Typically, the setup consists of a high DC voltage source (1–30 kV), a blunt ended needle or capillary of stainless steel, a syringe pump, and a flat or drum-shaped collector which is grounded. In case of electrospinning, polymer solution after gaining free charge from applied voltage when come at the tip of the capillary, a droplet forms on which two electrostatic forces, namely, repulsive force between like charges and Coulomb force due to external applied voltage, act. Due to these two electrostatic forces, droplet gets distorted into conical shape, known as Taylor cone. When electrostatic force counteracts surface tension, droplet gets injected from Taylor cone toward collector. Uneven distribution of charge on droplets causes whipping or bending motion of the jet and consequently elongation of jet, and raid evaporation of the solvent takes place, resulting in a solid thin fibrous structure on the collector as randomly oriented nonwoven mat. In contrast to electrospinning process, electrospraying technique relies on liquid atomization by electrical force. The parameters, by which final structure could be tuned, are molecular weight and microstructural features of the polymer, concentration of polymer, solvent, pH, conductivity, and viscosity surface tension of the solvent. Additionally, tip-collector distance, electric potential, and flow rate of solution affect fibrous structure.

10.8 Mechanism of Preservative Action

10.8.1 Protection Against Biological Deterioration

10.8.1.1 Antimicrobials

Microbial contamination has been leading to pathogenic infections and poor nutrition associated with weaning foods. Thus, dealing with bacterial deterioration is one of the most critical subjects in the production, processing, transport, and storing of food. Novel nanoantimicrobials have shown promising effects on safeguarding food deterioration, thereby extending the shelf life of food. A number of metal and metal oxide nanomaterials have long been suggested to be effective as antimicrobials. Their intrinsic physicochemical properties allow excessive formation of reactive oxygen species (ROS), leading to oxidative stress and subsequent cell damage. Furthermore, the release of metal ions outside the cell, at the cell surface, or within the cell can alter cellular structure or function (Prasad et al. 2016; Rajpal et al. 2016). Thus, metal/metal oxide-based nanocomposites have been utilized in food packaging and coating or even as ingredients. Silver nanoparticles and nanocomposites are one of the most widely used nanomaterials, as antimicrobials, in the food industry. Silver nanoparticles likely serve as a source of Ag ions, binding to membrane proteins, forming pits, causing other morphological changes, and catalyzing the generation of ROS in bacterial cells, subsequently leading to cell death through oxidative stress (Aziz et al. 2014, 2015, 2016, 2019). Nevertheless, multiple latest research studies suggested that silver nanocomposites are safe for food packaging, with no detectable or insignificant levels of silver nanoparticles that are released and migrated from impregnated containers into real food samples and food simulants. Nanocomposites offer added stability, which is important for sustaining antimicrobial activity and reducing the likelihood of migration of metal ions into stored foods. Polymers are largely engineered to form nanocomposites with metal/metal oxide nanomaterials for food application. Earlier, chitosan, polystyrene, polyvinylpyrrolidone, and poly(vinyl chloride) have also been reported as nanocomposite films that bind to Cu or ZnO nanomaterials to inactivate food pathogens.

10.8.2 Protection Against Chemical Ingredients

10.8.2.1 Antioxidants

Although some metal/metal oxide nanomaterials are known to cause oxidative stress via formation of ROS, less reactive nanomaterials are developed to act as antioxidant carriers. Polymeric nanoparticles are suggested to be suitable for the encapsulation of bioactive compounds (e.g., flavonoids and vitamins) and to release them in acidic environments (i.e., stomach). Furthermore, application of antioxidant treatments in association with edible coating is the most common way to control browning of fresh-cut fruits. It is well-known that browning of fresh-cut fruits is an undesirable effect brought by the conversion of phenolic compounds into dark-colored pigments in the presence of O_2, during storage and marketing. However, there are only a few applications of nanomaterials directly as antibrowning agents. Nano-ZnO-coated active packaging has been reported to be a viable alternative to common technologies for improving the shelf life properties of apples as a fresh-cut product.

10.8.2.2 Flavors

Nanoencapsulation techniques have widely been used to improve flavor release and flavor retention and to deliver culinary balance. It has also been shown that SiO_2 nanomaterials can act as carriers of fragrances or flavors in food and nonfood products.

10.8.2.3 Anticaking Agents

SiO_2 is used mainly to thicken pastes, as an anticaking agent to maintain flow properties in powdered products and as a carrier of fragrances or flavors in food and nonfood products. Recent research showed that in powdered food materials, at least a part of the SiO2 is in the nanosize range.

10.8.2.4 Others

In addition to the abovementioned benefits, nanomaterials have also been constantly developed to enhance the physical and mechanical properties of packaging in terms of tensile strength, rigidity, gas permeability, water resistance, flame resistance, etc. Aimed at providing those aforementioned properties, polymer nanocomposites are the latest materials with a huge potential for application in the active food packaging industry. Polymer nanocomposites with layered silicates were introduced in the 1990s. As these new polymer nanomaterials are much stronger, more flame resistant, as well as having a potential role in UV shielding applications, they are widely reported to have the potential to completely transform the food packaging industry.

10.9 Fate of Nanomaterials Within Human Systems

The nanoparticles found in foods may consist of inorganic and/or organic components. Nanoparticle composition plays a major role in determining their GIT fate as has been summarized in Table 10.1. Lipids, proteins, and starches can be digested by proteases, lipases, and amylases in the mouth, stomach, small intestine, or colon.

Table 10.1 Nanoformulations and their impact on human health

Nanoemulsion	Component	Health hazards
Nondigestible inorganic nanoparticles	Silver nanoparticles	Reducing ATP content Increasing ROS Production Damaging mitochondria and DNA chromosomal aberration Genotoxic Cytotoxic Carcinogenic
Digestible organic nanoparticles	Surfactants	Bioaccumulation
	Lipids, proteins, and carbohydrates	Cellular damage Degradation of proteins Cardiovascular diseases
	Carbon nanotubes	Obesity
		Cause skin and lung disease

However, some organic substances used to fabricate food nanoparticles (such as dietary fibers and mineral oils) may not be digested in the upper GIT. Inorganic nanoparticles are also not digested in the GIT, but some of them may be fully or partially dissolved as a result of alterations in pH or dilution. Any nanoparticles that are not digested or absorbed in the upper GIT will reach the lower GIT where they may alter the microbiome. The ability of inorganic nanoparticles to produce toxicity is often associated with their chemical reactivity, which depends on their composition. Nanoparticle dimensions influence their GIT fate and toxicity through a number of mechanisms. The GIT fate of food-grade nanoparticles, and therefore their potential to have adverse health effects, is often influenced by their interfacial characteristics. Food-grade nanoparticles may exist as isolated individual particles, or they may form clusters that vary in size, morphology, and strength. Typically, nanoparticles in clusters are held together by physical forces, such as Van der Waals, electrostatic, hydrogen bonding, and hydrophobic forces. The aggregation state of the nanoparticles is therefore often highly dependent on environmental conditions, such as pH, ionic strength, ingredient interactions, and mechanical forces. The dimensions of nanoparticle clusters may be much greater than the dimensions of the individual nanoparticles, which has a major impact on their GIT fate, such as their ability to move through the gastrointestinal fluids, mucus layer, or epithelium cells. Consequently, it is always important to determine the actual effective dimensions of the nanoparticles at the site of actions, rather than the dimensions of the original nanoparticles added in foods.

10.10 Conclusion

Embracing the development of nanotechnology and its application in food industry specifically for preserving the food quality attributes is likely to witness both success like in other fields and also certain implications of concern if used indiscriminately. Although the fate and potential toxicity of nanomaterials are not fully understood at this time, it is evident that there have been significant advances in the application of novel nanotechnology in the food industry. Application of nanotechnology can also assist in the detection of pesticides, pathogens, and toxins, serving in the food quality tracking-tracing-monitoring chain. Furthermore, nanotechnology has the potential to bring in a radical transformation in future food packaging systems. However, the challenges to develop a healthy and sustainable food industry remain with the personnel and organizations applying this nanotechnology taking precautions with respect to associated health, safety, and environmental impacts be addressed and regulated at the forefront. To be successful in the long run, proper education of the public is also paramount in the introduction and development of nanotechnology in food system.

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Chapter 11
Sensing Soil Microbes and Interactions: How Can Nanomaterials Help?

Poonam Sashidhar, Mukul Kumar Dubey, and Mandira Kochar

11.1 Introduction

A tremendous diversity of microbes resides in and around plants as the plant microbiome. They form multiple and interacting ecological communities that significantly influence plant growth and health through their collective metabolic activities and host interactions. The interactions of the plant microbiome with environment are known to cause a substantial impact on soil and human health. Rhizosphere, the narrow zone of soil that surrounds the root system, is influenced by plant roots (in association with root hairs and plant-produced materials) and comprises a cornucopia of organisms, including bacteria, fungi, protozoa, archaea, and viruses (Dessaux et al. 2009; Walker et al. 2003; Shrivastava et al. 2014; Mueller and Sachs 2015). Plant microbiomes play a key role in improving plant growth, health, and productivity besides giving protection to the plants from phytopathogens offering an overall adaptive advantage (Berg et al. 2015; Haney et al. 2015; Prasad et al. 2015). Thus, the plant microbiome plays a constructive role in sustainable agriculture with the bacteria garnering more attention, compared to the other groups of organisms. As a part of the plant microbiome, the naturally existing soil microbes are considered responsible for key ecosystem services vital for life, including nutrient cycling and sustainable plant growth (Berg et al. 2015; Singh et al. 2019).

Plants are colonized by a vast number of microbes that could be more than the number of plant cells. In addition, there are also a large number of microbial genes in the microbiome than plant genes, and this comprises the plant's other genome as well. There is a vast amount of literature confirming the plant-associated microbes to possess immense plant-beneficial functions and traits, such as their profound

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effects on seed germination, seedling growth, plant development, nutrition, diseases, and productivity (Fierer 2017; del Carmen Orozco-Mosqueda et al. 2018). In return, plants deposit their photosynthetically fixed carbon into their immediate surroundings, i.e., the spermosphere, phyllosphere, rhizosphere, and mycorrhizosphere (Berendsen et al. 2012; Raaijmakers et al. 2009; Vorholt 2012; Giri et al. 2005), thereby feeding the microbes in the microbiome and consecutively influencing their composition as well as activities. The plant-microbe interactions have been studied in depth, but there is limited knowledge of their impact on plant growth, health, and soil processes. Soil particles and pores create microhabitats that act as niches for different microbial communities (Ruamps et al. 2013; Rabbi et al. 2016). To understand the impact and significance of microbes on the plant microbiome and soil, the microbes need to be detected in soil niches and traced through soils. This chapter will highlight the different strategies that may be utilized for detecting microbes (especially bacteria) of the microbiome and their interactions in soil.

11.2 Microbiome

Successful detection and modulation of the plant microbiome bacteria enable scientists to not only influence plant beneficial activities but also favorably influence soil health by reducing the use of chemical fertilizers. Plant-produced materials, referred to as root exudates, play a major role in shaping up the microbial communities around the root surface and are diverse compounds, including organic acids, enzymes, amino acids, and complex carbohydrates, synthesized by plant roots that act as chemical attractants for soil microflora (Ahemad and Kibret 2014; Walker et al. 2003). The root tips aid in bacterial colonization by providing steady-state redox conditions and an ambient structural surface (Wu et al. 2006). The soil bacterial community that competitively colonizes plant roots is termed as rhizobacteria (Kloepper 1978). Rhizobacterial interaction with plants is classified into three categories: (i) neutral, (ii) negative, and (iii) positive. Certain rhizobacteria establish innocuous interaction with plants, showing no visible effect on growth (Beattie 2007). During negative interactions, the rhizobacteria, by virtue of production of phytotoxic compounds such as hydrogen cyanide and ethylene, negatively influence growth and physiology of the plant (Bhattacharyya and Jha 2012; Kamal et al. 2010). On the other hand, there exist plant growth-promoting bacteria (PGPB) which stimulate plant growth by utilizing various direct and indirect mechanisms. PGPB are identified on the basis of three distinctive features: (i) aggressive colonization of the root surface; (ii) ability to survive, multiply, and compete with other soil microbiota; and (iii) plant growth stimulation by various direct and indirect mechanisms (Bhattacharyya and Jha 2012; Kloepper 1994; Vessey 2003). Additionally, these strains may display biocontrol properties for combating pathogenic organisms (Bhattacharyya and Jha 2012). Based on their mode of colonization, PGPB can be subdivided into extracellular and intracellular PGPB (Martínez-Viveros et al. 2010). The extracellular PGPB (ePGPB), including

Azotobacter, *Azospirillum*, *Bacillus*, *Burkholderia*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, and *Serratia* exist in rhizosphere, rhizoplane (root surface along with strongly adhered soil particles) or between cells of the root cortex. On the other hand, intracellular PGPB (iPGPB), including strains of *Frankia* and other endophytes such as *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*, colonize specialized nodular structures (Bhattacharyya and Jha 2012; Ahemad and Kibret 2014).

The direct growth-promoting mechanisms include biological nitrogen fixation, production of phytohormones (such as auxins, cytokinins, and gibberellins) as well as plant growth regulators, solubilization of key plant nutrients like iron and phosphorus, and siderophore production. On the other hand, indirect mechanisms comprise the biocontrol pathways, including antibiotic production, secretion of fungal cell wall-degrading enzymes, etc. (Bhattacharyya and Jha 2012; Ahemad and Kibret 2014). Based on their mode of action, PGPB can be broadly classified into three groups: phytostimulants, biofertilizers, and biopesticides. A detailed listing may be found in Table 11.1.

11.3 Soil Microbial Sensing

During plant colonization and in the rhizosphere, many microbes and the microbial communities modulate the development of plants and influence the root system architecture to a great extent. The beneficial effects of microbes in the rhizosphere are the result of complex interactions among all rhizosphere microbes, and this is crucial for plant growth (Bonfante and Anca 2009). Microbial attachment on surfaces (biotic or abiotic) in soil results in the formation of biofilms and communities that can have a greater impact on natural processes. The three-way interaction between plants, fungi, and microbes in soil are of great importance for sustainable agriculture. Further, in changing climates and suboptimal farming conditions, the performances of microbes and bioformulations tend to be inconsistent along with variations in bacterial community dynamics. Thus, it becomes important to understand these microlevel changes among naturally existing communities if novel bioformulations are to be developed and validated for supplementation of existing agricultural practices.

Microbial diversity in soil is measured by various techniques such as traditional plate count method or direct count, molecule-based platforms, and conventional sensors. There is a range of molecular techniques to detect, to characterize, and/or to type bacteria, and these can be applied to soil bacterial communities, and these include cultivation-dependent and cultivation-independent methods such as PCR-based fingerprinting, quantitative PCR (real-time), probing with isotopes, and in situ hybridization with fluorescence probes (Schmidt and Eickhorst 2014). Direct DNA extraction and PCR from soil samples are also routine strategies to study microbial populations coupled with techniques like denaturing gradient gel

Table 11.1 Nature and function of plant growth-promoting bacteria

Behavioral type	Function	Examples	References
Phytostimulants	Production of phytohormones and growth regulators (auxins, cytokinins, gibberellins, and nitric oxide)	*Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Brevibacillus*, *Rhizobium*, *Sphingomonas*, *Serratia*, *Xanthomonas*	Noel et al. (1996), Verma et al. (2001), Ma et al. (2011), Tsavkelova et al. (2005), Selvakumar et al. (2008), Jiang et al. (2008), Sheng and Xia (2006), and Creus et al. (2005)
	Decreased ethylene concentration (ACC-deaminase activity)	*Stenotrophomonas maltophilia*, *Rahnella aquatilis*, *Enterobacter* sp., *Burkholderia*, *Pseudomonas* sp.	Rajkumar and Freitas (2008), Jiang et al. (2008), Kumar et al. (2008), and Mayak et al. (1999)
Nutrient mobilizers/ biofertilizers	Biological nitrogen fixation	*Klebsiella*, *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azospirillum*	Jha and Kumar (2007), Vessey (2003), Wani et al. (2007), and Rodrigues et al. (2008)
	Phosphate solubilization	*Klebsiella*, *Pseudomonas*, *Bacillus*, *Enterobacter*, *Acinetobacter*, *Stenotrophomonas*	Jha and Kumar (2007), Wani et al. (2007), Kumar et al. (2008), and Rokhbakhsh-Zamin et al. (2011)
	Metal accumulation	*Bradyrhizobium*, *Gluconacetobacter*, *Brevibacillus*, *Pseudomonas* sp., *Xanthomonas*, *Azomonas*	Dary et al. (2010), Ma et al. (2010), Saravanan et al. (2007), Vivas et al. (2006), Rajkumar and Freitas (2008), and Sheng and Xia (2006)
Biopesticides	Production of antibiotics (siderophores, HCN, antifungal metabolites)	*Bradyrhizobium*, *Pseudomonas*, *Ralstonia metallidurans*, *Paenibacillus*; *Acinetobacter*, *Azotobacter*	Braud et al. (2009), Tank and Saraf (2009), Wani et al. (2007), Zahir et al. (2010), and Indiragandhi et al. (2008)
	Production of enzymes that degrade the cell wall of fungi	*Serratia*, *Chromobacterium*, *Paenibacillus*	Kim et al. (2008), Frankowski et al. (2001), and Singh et al. (1999)
	Acquired and induced systemic resistance	*Paenibacillus polymyxa*, *Pseudomonas*	Chandler et al. (2008), Phi et al. (2010), and Saravanakumar et al. (2007)

electrophoresis (DGGE), amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP) (Picard et al. 1992). This can give a detailed view of the different bacterial communities present in soil, provided the signal strength and autofluorescence do not result in interference.

11.3.1 Plate and Direct Count Method

In the past, microbial population in the soil had been detected by plate counts or the most probable number (MPN) technique. The cultural diversity has been assessed precisely by isolating the colonies from the media followed by various biochemical characteristics. The major drawback of determining the microbial population in soil is its laborious procedures and the identification of only the culturable microbes. The recent molecular investigations do not rely on the isolation-cultivation technique (Johnsen et al. 2001). Small bacterial cells are not culturable due to their inability to form colonies in agar medium. The direct count by fluorescence microscopy identifies more microbes than the plate count method. Proteins or nucleic acids of the microbial population have been stained with specific fluorescent stains like fluorescein isothiocyanate (FITC), ethidium bromide, and acridine orange. This process however does not differentiate between live and dead cells and specific microbial cells.

11.3.2 Molecular Techniques

Molecular techniques involve extraction of DNA from the soil microbial population directly or indirectly. They are sensitive in detecting taxonomic groups and independent of culture. Measurement of base distribution in nucleic acids, determination of rates at which the single-stranded DNA reanneals at low temperature below its melting point, is categorized in low-resolution molecular techniques (Torsvik 1994; Torsvik et al. 1996). High-resolution techniques involve polymerase chain reaction (PCR), ribosomal interspace analysis (RISA) based on polymorphism in the 16S and 23S rRNA spacer regions, and random amplification of polymorphic DNA (RAPD). Change in the microbial response with respect to nutrient addition to soil was monitored using bromodeoxyuridine-labeled DNA isolated from soil microbes using RISA (Borneman 1999). Microarray of DNA is a powerful tool for analyzing vast microbial communities as it contains 500–1000 different DNA probes (Tiedje et al. 2001). A successful molecular technique is based on an efficient isolation of DNA and its purification. The isolation of DNA involves an in situ lysis (direct) or initial cell extraction before isolation (indirect). The third category of molecular technique is the one offering an intermediate resolution. Denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) is the most widely used characterization technique for microbial communities in soil, namely, the hydrothermal vents and the hot springs (Liesack et al. 1991). Although widely accepted, they possess a drawback of revealing only the dominant populations. More than one species can show a single band, thus underestimating the bacterial diversity. DGGE and TGGE are now used to create fingerprints of fungal 18S rRNA because of the availability of specific primers (Nannipieri et al. 2003).

11.3.3 Optical Methods

Optics has played a crucial role in development of biosensor and shaping it as a fundamental tool for sensing the target signals. One example of optical biosensor is the surface plasmon resonance (SPR) (Ziegler and Göpel 1998). SPR determines the target analyte without the need of labeled probe. With the integration of modern technologies, the applicability of conventional optical biosensors has increased. In particular, integration of microwell plates with luminometer that quantifies the intensity of luminescent light has been widely adopted for experiments employing luminescence-based biosensors and requires measuring the activity of luciferase (Lim et al. 2015). Bioluminescence is a time-consuming and highly sensitive method for bacterial detection. The assay is based upon the principle of living cell of a particular species that contains a reasonably constant amount of adenosine 5'triphosphate (ATP), which is lost upon cell death. "Lumac Biocounter" (Netherlands) and "Unilite" (Bridgend, UK) are the two commercially available bioluminescent sensors for microbial detection. Both these bioluminescent sensors can detect low levels of microbes (10^3 ml^{-1}) from the swab without any dilution losses (Hobson et al. 1996).

In the past few years, a lot has been unraveled about the abundance of different microbes and the functional and metabolic relevance of the plant- and soil-associated microbiome, but it has become increasingly important to understand the genomic and metabolite diversity existing in soil with reference to individual microbial species. This will help toward using soil microbial functions for the purpose of sustainable agriculture (Mauchline and Malone 2017). Climatic and other abiotic factor variations profoundly influence the expression of genes and proteins in the soil microbial population, thereby indirectly affecting their abundance (Parejko et al. 2012; Mauchline et al. 2015). Additionally, the microbial community with high taxonomic diversity and functional/metabolic potential can be an appropriate biological indicator of ecological processes in operation. Hence, to measure the specific microbial populations in soil, it is fairly important to understand their contributions and functional processes in soils (Mendes et al. 2013). However, obtaining accurate population density and functional information of soil microbial species has not been a very successful activity in the past due to the inability to capture such microbes and microbial activity in vivo (Mauchline and Malone 2017). Coupled to this is the nonculturability of many of the soil microbes that skews the information generated from soil microbial community analysis toward only the culturable microbes. With the rapid development of innovative genomics and transcriptomics strategies and high-throughput molecular technologies, previously unknown species are being discovered, and functions can now be annotated to previously hypothetical genes and proteins. With the development of culture-independent measurements through advances in metagenomics, various approaches, including counts of bacteria, their genetic assessment, and biochemical measurement, have been used to quantify the microbial numbers, and novel sensors can be developed to track the presence of such microbes.

Metagenomics strategies can be used for discerning species identity and for detecting interspecies association patterns. Microorganisms can utilize and secrete a large number of metabolites in response to nutrients and climatic and environmental changes including abiotic and biotic factors (Barve and Wagner 2013; Gallie et al. 2015). Metabolome and metabolite analyses influence the structure and spatial spread of the microbial communities and metabolic processes, and gene and/or proteins expressed can be used as indicators of the presence of known microbes in unknown samples. For instance, individual genomes of an associated archaebacteria showed that one of the symbionts is dependent on the other for metabolites such as lipid, cofactors, and amino acids (Waters et al. 2003). If this is additionally supported by data retrieved by genotyping of associated microbes, the metabolites can be used to develop tracking strategies for the specific bacteria.

11.4 Innate Microbial Nanowires as Sensors

Microorganisms can on their own also produce sophisticated nanomachines like bacterial flagellar nanomotors made up of numerous, innate proteins (Chalmeau et al. 2009). Such proteins are being used as building blocks for the construction of nanodevices including sensors and drug-delivery vehicles (Petrov and Audette 2012; Scanlon and Aggeli 2008; Rosenman et al. 2011). Peptide nanotubes have been developed and can be used as a casting module or biotemplate for synthesis of metal nanowires (Reches and Gazit 2003; Scanlon and Aggeli 2008). Most proteins made of natural amino acids are insulating (Scanlon and Aggeli 2008), and thus efforts were made to build electrically conductive protein nanotubes which in turn can act as nanowires (Scanlon and Aggeli 2008; Creasey et al. 2015). Such nanowires have immense application in nanoelectronics (Lovley 2017). Extracellular electrically conductive protein nanofilaments or microbial nanowires (MNWs) have in the last decade or so been discovered in numerous anaerobic and aerobic microorganisms (Reguera et al. 2005). Their mechanistic aspects and functional relevance have been delved upon by many world-renowned research groups, and this research has opened many new applications of research especially in sensing and detoxification of metals.

MNWs can facilitate electron transfer between microbial cells producing them and the extracellular electron acceptor/donors in their environments. In metal-reducing bacteria like *Geobacter sulfurreducens*, MNWs can help transfer electrons to metals which act as electron acceptors even though they may be available at a distance, without the need of direct cell attachment or dissolved electron shuttles (Reguera et al. 2005). In the metal-oxidizing bacteria *Acidithiobacillus ferrooxidans*, MNWs possess the ability to transfer electrons to the cell surface, allowing the accessibility of electron donors at a distance (Li and Li 2014). In anaerobic environments, MNWs of photosynthetic bacteria can bridge the gap between the cells and use arsenic as an electron donor (Kulp et al. 2008). *Synechocystis* MNWs have shown that they can bind and immobilize arsenic, thereby acting as a conduit of electrons between the cyanobacterial cells and arsenic (Sure et al. 2016a). Due to their capability to interact with metals, MNWs can serve as a protective cellular mechanism against sensing and detoxifying toxic metals (Figs. 11.1 and 11.2). Appropriate large-scale studies are however required to be done to allow remediation and sensing studies.

11.5 Nanomaterials for Microbial Sensing

Biosensor-based strategies involve a probe (recognition element) that binds to the target analyte and transducer to convert the signal into detectable forms. However, the wide-scale applicability of biosensors is limited by some factors like specificity, dynamic range of detection, signal-to-noise ratio, and detection limits. Nanomaterials conjugated with such probes or recognition elements can improve the biosensor performance by making the recognition and transduction process more specific (Saha et al. 2012). Several nanoparticles have been exploited in the construction of biosensor as probes to interact with the target analyte or as transducers to measure the binding event and quantify the signals. Nanomaterials that have been investigated for the detection of microbes include carbon nanotubes (CNTs), metal nanoparticles (mNPs), quantum dots (QDs), silica nanoparticles, and upconversion

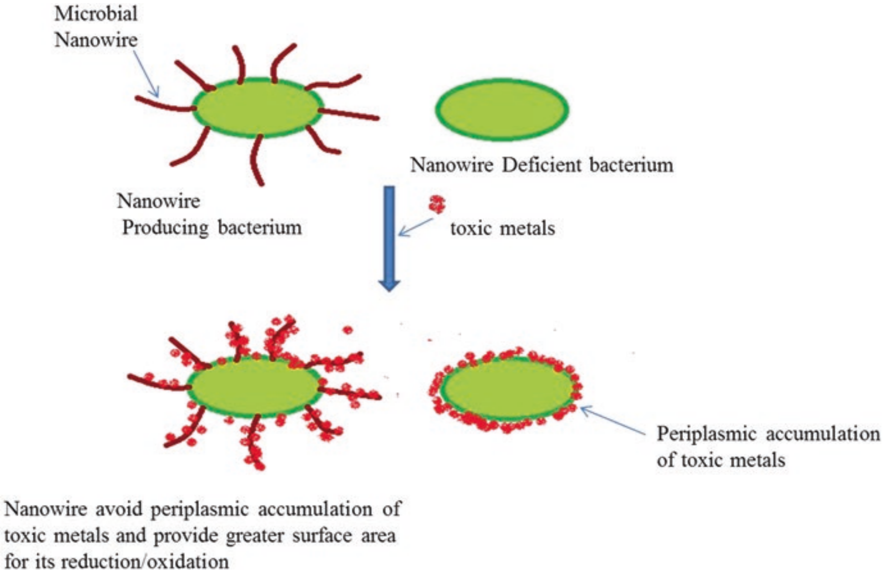

Fig. 11.1 Potential role of microbial nanowires in microbial cell-metal interactions and metal sensing. Schematic representation of how microbial nanowires can sense toxic metal presence and reduce interaction of toxic metals and cell membrane, protecting the microbial cells. (Reproduced from Sure et al. 2016b)

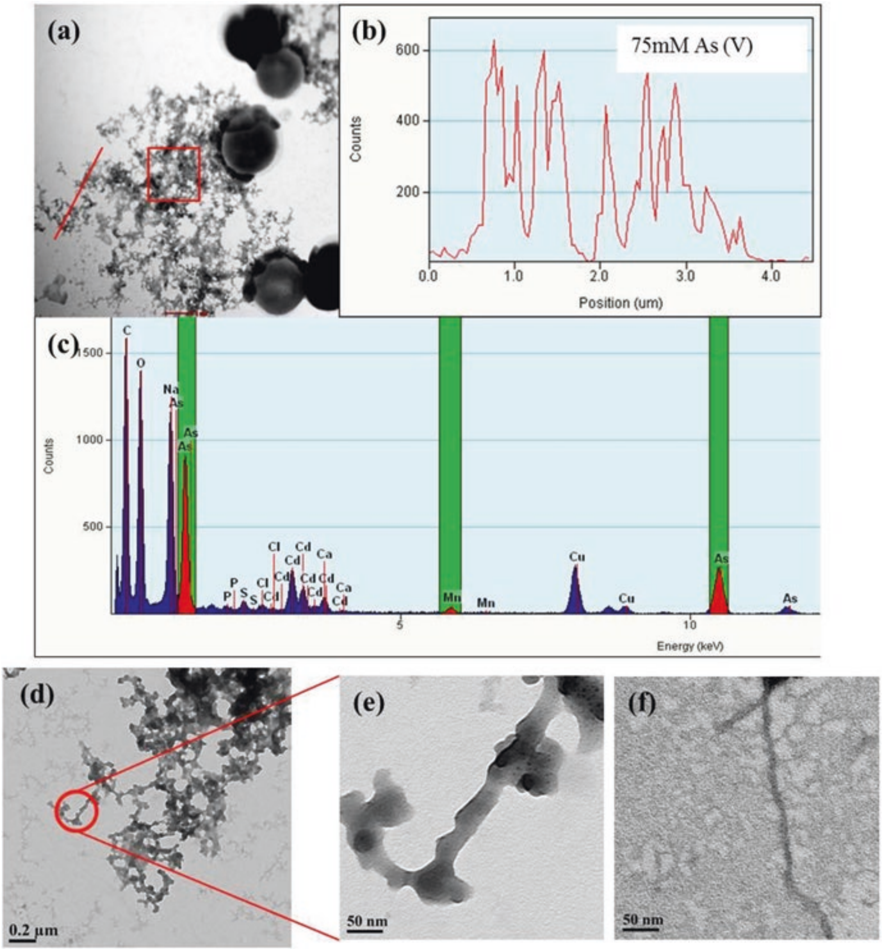

Fig. 11.2 Metal (arsenic, As) binding to microbial (*Synechocystis*) nanowires. Microbial cells on exposure to minimum inhibitory concentration of As (75 mM As) (**a**). Line EDX spectra (**b**) and EDX of square area (**c**) (highlighted by red color in a) confirm the presence of As on putative MNW-As complexes. EDX spectra (**c**) show small Mn peaks suggesting its possible presence on putative MNW. (**d**, **e**) High-magnification images of putative MNW-As complexes (≥15 nm diameter). MNW from control cells (5–7 nm in diameter) (**f**) at the same magnification (×29000) as that of putative MNW-As complex in image (**e**). (Reproduced from Sure et al. 2016a)

nanoparticles (UCNPs). These materials are easily amenable to nanosized structures, conferring superior properties specific for biosensing applications (El-Ansary and Faddah 2010). The attachment of recognition elements to nanoparticles or nanoprobes is a crucial step that determines the functionality of a biosensor. The attachment can be carried out by various mechanisms like physical adsorption, covalent bonding, bioconjugation, etc. (Park et al. 1999). From the methods listed above, attachment using covalent linkages offers a robust connectivity by the strong amide linkages. In addition to the size and surface reactivity, nanoparticles exhibit surface plasmon resonance (SPR), quantum confinement, and superparamagnetism making them ideal for constructing next-generation biosensors. Specific research groups are working exclusively on incorporating the SPR phenomenon of the metal nanoparticles for biosensor development. These metal nanoparticles can be tailored to exhibit a characteristic SPR pattern based on their size and composition (Taton et al. 2001).

Nanoparticle-conjugated systems identify the bacterial cells using genotypic and phenotypic mechanisms. A study which focussed on the genotypic identification of bacteria used Fe_3O_4 nanoparticles to form a quartz crystal microbalance (QCM) DNA sensor. This DNA sensor comprised of a DNA probe complementary to the target DNA of the bacterial cell. Upon recognition of the target DNA, followed by hybridization, the nanoparticles were used to bind to the target DNA as "mass enhancers" and amplify the frequency change (Mao et al. 2006). Phenotypic identification uses the morphological and biomarker difference between the bacterial cells. For instance, NPs bound to a specific biomarker can specifically bind to a type of bacteria and offer rapid detection. To support this phenomenon, a research group used the anti-*Escherichia coli* and anti-*Salmonella typhimurium* antibodies to specifically bind to their target organisms and separate the individual organisms from the consortium (Ravindranath et al. 2009). Novel systems harnessing nanosensors are developed for mycotoxin analysis progressing toward precision agriculture ensuring safe food surveillance. Some metallic NPs like Ag, CuO, and ZnO are known to attack the bacterial membranes. For example, short-time exposure of *E. coli* to nano-ZnO results in the loss of cell membrane integrity. The control of pathogenic microbes in the soil by antimicrobial NPs is an important approach that can be used for biosensor development (Gajjar et al. 2009). In addition to the single probe sensors, nanochips composed of microarrays immobilized with target probes were used to detect the presence of bacteria and viruses based on a single nucleotide change. Nanosensors could be directly placed on sites where they would detect chemical changes during a soil pathogen attack and control their proliferation. Thus, the monitoring of soil microbes using nanosensors can provide insights of crop health and soil conditions. Precision farming will enhance the agricultural practices by providing precise data and to effectively adopt appropriate measures (Rai and Ingle 2012; Prasad et al. 2014, 2017a, b).

Sensors that use nanoscale materials possess capabilities to detect specific functionalities through donor-acceptor interactions or utilizing spatial recognition through cavities, grooves, and channels of the host material (Dickert and Haunschild 1993). Nanobiosensor applications in agriculture are expected to deliver real bioanalytical data, which were earlier difficult (Rai et al. 2012). Nanomaterials interact with microbes and their communities through applications of pH-sensitive polymers or defined nanostructures and can be employed for evaluating these interactions. Present research on nanobiosensors for agricultural applications aims to develop effective monitoring applications for soil quality with respect to constituents, pH, humidity, and microbial flora that could help in improving agricultural yields alongside the applications to plant systems (Kwak et al. 2017; Sangeetha et al. 2017). Similarly, depending upon protein-ligand interactions, protein-nanoparticle-based biosensors are being explored for ultrasensitive detection of differential protein molecules. The use of these nucleic acid- and protein-detecting biosensors is suggested to be important in microbes and pathogen detection among other valuable functions. Different types of biosensors that can be used for microbial detection and sensing have been developed and are listed below:

- Nanosensors that use single-stranded DNA-carbon nanotubes (single-stranded DNA-CNTs probes) or recognition element-nanomaterial complexes or other nanoprobes for microbial detection. They find application in visual detection of species-specific DNA oligonucleotides (Cao et al. 2008) for diagnostics or identification of transgene.
- MWNTs/ZnO/CHIT composite film modified GCE for immobilization of ssDNA probes to effectively discriminate different DNA sequences (Zhang et al. 2008a, b).
- Nanobiosensor with gold nanoparticles functionalized with alkane thiol-capped LNA/DNA chimeras as hybrids, for single-stranded DNA (McKenzie et al. 2007). Nano- SiO_2 functionalized with p-aminothiophenol have also been designed and used to detect the presence of p-aminothiphenol gene sequences following a label-free method (Ma et al. 2008).
- Nanosensors with a core-shell structure and loaded with appropriate fluorescent dyes help in the detection of biofilms as they can sense minute changes in biofilm microenvironment based on pH change (Hidalgo et al. 2009).
- "Chemical nose" nanosensors that can aid in the quantitative detection of bacterial presence through change in colorimetric response due to the aggregation of nanoparticles (Verma et al. 2014, 2016).
- Nanowire field-effect transistor-based biosensor to attain simple and ultrasensitive DNA methylation detection alongside avoiding complicated bisulfite treatment and PCR amplification (Maki et al. 2008).
- Protein nanoparticles can be used for the ultrasensitive detection of protein molecules using specialized protein-ligand interactions. The use of such protein-detecting biosensors might play a vital role in the detection of phytopathogens and development of biomarkers.
- Photonic nanomaterials (e.g., surface-functionalized, hydrophobic graphene quantum dots) may also be a useful alternative to detect the presence of microbes through microscopic and spectroscopic techniques.
- Single-walled carbon nanotubes (SWNTs) can passively transport and irreversibly localize themselves within the lipid envelope of plant chloroplasts and in the process promote photosynthetic activity and electron transport rates in plant chloroplasts (Giraldo et al. 2014).
- Reactive oxygen species can also be significantly suppressed by delivering nanoceria or SWNT-nanoceria complexes to chloroplasts. Near-infrared fluorescence monitoring of nitric oxide produced in ex vivo and in vivo conditions demonstrated that a plant can be made to function as a photonic chemical sensor (Giraldo et al. 2014).

It still remains to be seen whether some of these sensing technologies can be applied to microbes, especially those present in soil, but given the parallels and commonalities in biomolecules (and their functions) between plants and microbes, it is likely that such sensors may be used in the future for the detection and sensing of microbes on a large scale.

11.6 Nanomaterial-Microbe Interactions

11.6.1 Materials Used

11.6.1.1 Metal Nanoparticles

To begin with, metal nanoparticles like gold and silver with varying shapes and sizes possess intrinsic properties like localized surface plasmon resonance (LSPR) and different Raman scattering intensities depending on electromagnetic field enhancement. With a change in the ambient dielectric constant, a visible color change is caused resulting from particle aggregation. Metal nanoparticles have been utilized as colorimetric and SERS probe, FRET acceptor, and fluorescence enhancer and to quench fluorescence (Sutarlie et al. 2017). With the properties listed above, gold nanoparticle-based optical sensors have been developed, and the SPR phenomenon has been exploited in detecting karnal bunt disease in wheat (Singh et al. 2010). Gold nanoparticles have also been used in the construction of probes for the identification of pathogenic bacteria using DNA-microarray technology (Taton et al. 2000). In the case of the gold nanoparticles, the color shift from red to blue caused by particle aggregation can be easily visualized by visible light. It does not require fluorescence excitation or emission spectrum, ultimately resulting in significant cost reduction. Antibody-conjugated gold nanoparticles have been used in the colorimetric detection of *E. coli* O157:H7 (Singh et al. 2009). As the bacterial cells are larger than the nanoprobes attached to them, several of them can attach to the surface of the bacterial cell and result in color shifts (Fig. 11.3a). The thiol groups present in the gold nanoparticles can be modified with ssDNA which can be used as tags in identification of complementary microbial sequences. Gold nanoparticles have already been used in multiple formats for the detection of bacterial cells. For example, the positively charged gold nanoparticles bind to negatively charged protein surfaces through electrostatic attraction. The nanoparticles can interact with the enzymes, inhibiting the enzyme activity. A study reported the reversal of enzymatic activity by positively charged polyethyleneamine-coated gold nanoparticles to detect bacterial cells in drinking water by optical methods (Fig. 11.3b).

11.6.1.2 Quantum Dots

Quantum dots (QDs) carry semiconductor properties and are typically composed of core shell structure. They are fluorescence-emitting nanoparticles due to the quantum confinement governed by the semiconducting band gap. QDs have better

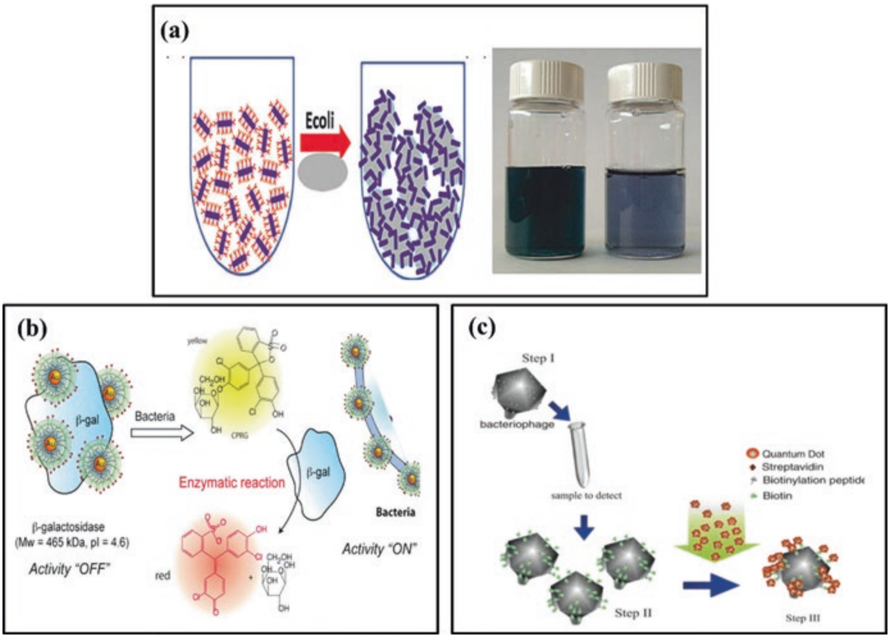

Fig. 11.3 (**a**) Schematic representation of colorimetric detection of *E. coli* bacterial cells using anti-*E. coli* antibody-conjugated gold nanorods (left) and photograph of the color changes before and after adding *E. coli* (right). (**b**) Bacterial detection using electrostatic interaction between positively charged AuNPs and negatively charged enzyme (**c**) T7 phages labeled with quantum dots via streptavidin-biotin interaction for bacterial detection. (Adapted and Reproduced from Chen et al. (2017) by Permission of The Royal Society of Chemistry)

photostability and broader absorption property which allows the excitation of multiple QDs by single wavelength and also narrow emission properties based on particle size allowing multiple detection systems for QDs (Algar et al. 2010; Kim et al. 2013). The coating of QDs allows efficient functionalization with various biorecognition units which can prevent toxicity (Biju et al. 2010). Gold nanoparticles are combined with QDs to increase the fluorescence owing to their high plasmonic behavior. Localization of gold nanoparticles around the surface of QDs increases the excitation of QDs, hence leading to increase in the fluorescence intensity (Maye et al. 2010). QDs are also used in combination with magnetic nanoparticles for improved detection of biomolecules. QDs and upconversion nanoparticles (UCNPs) conjugated with different aptamers were used to detect different molecules which served as recognition elements and transducers for short DNA strands coupled with magnetic nanoparticles (Kurt et al. 2016). A recent technique for identification of bacterial cells that have attracted a lot of researchers is the engineered phage-based detection (Fig. 11.3c). The reporter tags carrying phages are amplified eventually improving the detection of bacteria. Streptavidin-conjugated quantum dots were used to label biotin-labeled phages to identify *E. coli* through fluorescent quantification (Edgar et al. 2006).

11.6.1.3 Carbon Nanoparticles

Carbon is a material of choice for biosensing application due to excellent conductivity and biocompatibility (Kivirand et al. 2015). It is preferred for electrochemical signal transduction. The standard materials for the construction of electrodes in electrochemical sensing are glassy carbon, doped diamond, and graphite (Uslu and Ozkan 2007). Carbon nanotubes (CNTs) and graphene are widely used materials for biosensing application, while carbon dots (CDs) and graphene quantum dots (GODs) are new-age materials for photoluminescence (Himaja et al. 2015). It is reported that CDs and GODs are more ecofriendly than QDs and display low toxicity and can be easily synthesized from renewable materials like carbohydrates, bagasse, and fruit peels. CDs and GODs are biodegradable and can be used as fluorescent probes or FRET donors in biosensing application. Powerful FRET between gold nanoparticles and carbon QDs are proposed by modifying each nano-object with a corresponding antibody-antigen system (Bu et al. 2014). CNTs were bound to platinum (Pt) nanoparticles for enhanced DNA sensing using daunomycin. Daunomycin is a redox-active compound aiding in intercalation of DNA. The signal transduction was carried out by differential pulse voltammetric signals generated from the intercalation event of daunomycin to specific recognition element. The surface area of CNTs and the efficient catalytic properties of Pt elevated the performance of the whole setup (Zhu et al. 2005).

11.6.1.4 Magnetic Nanoparticles

Magnetic nanoparticles have been used for long as contrasting agents in magnetic resonance imaging and as immunomagnetic separators for nucleic acids, proteins, viruses, and bacterial cells (Abd-Elsalam et al. 2019). The magnetic nanoparticles have been coated with polymers like dextran and silica to enhance their water solubility. The surface modification of magnetic nanoparticles can facilitate more conjugation to the recognition elements by the introduction of carboxylic acids and amino acids. A study was carried out to investigate the use of superparamagnetic silicate-magnetite nanoparticles for the isolation and purification of DNA from the soil microbes (Sebastianelli et al. 2008). The alteration in the properties of magnetic nanoparticles resulting from their molecular interaction with the target DNA can be detected with magnetometers or superconducting quantum interference device (SQUID) (Kaittanis et al. 2010). In another study, superparamagnetic iron oxide nanoparticles were functionalized to identify *Mycobacterium avium* spp. The use of magnetic nanoparticles ensured specific binding to *Mycobacterium* spp. and sensitive detection of bacterial target in milk and blood (Kaittanis et al. 2007). The magnetic relaxometers are used in quantification and detection by monitoring the changes in the spin-spin relaxation time of protons present in solution due to the association of the nanoparticles with the target.

11.6.1.5 Upconversion Nanoparticles (UCNPs)

UCNPs are relatively a new class of nanoparticles which are luminescent in nature and consist of lanthanide-doped compounds. The doping of lanthanide compounds is combined with inorganic nanocrystals of size range less than 100 nm. UCNPs are employed as fluorescent probes or FRET donors. The UCNPs work on the principle of upconversion process where low-energy excitation (IR) is converted into a higher excitation state in near-infrared or visible spectrum at shorter wavelengths (Sutarlie et al. 2017). The color emitted from the UCNPs depends on the type of lanthanide dopant. The excellent optical properties of the UCNPs have enabled the research to develop composite systems which add to the sensitivity of detection. UCNPs conjugated to different aptamers were used for detection of a consortium of three microbes present in food and environment (Wu et al. 2014). However, the UCNP-based sensors have some limitations. The increase in the strains will interfere with the fluorescence intensity and can decrease the sensitivity of the kit. The other limitation attributes to the limited number of aptamers specific for a microbe (Abbasian et al. 2018).

11.6.2 Mechanistic Aspects

In the current era, nanoparticles have found their application in various applications like biomedicine, drug delivery, agriculture, environmental protection, biosensing, etc. It is however mandatory to understand the interaction of such materials with different biological systems before considering its utility for the abovementioned application (Moyano and Rotello 2011; Prasad 2014; Prasad et al. 2016, 2018). In a biological medium, nanoparticles interacting with biomolecules form nano-bio conjugates depending on their shape, surface properties, and size. The physicochemical properties of the nanoparticle and biomolecule will govern the interaction at nano-bio interface (Nel et al. 2009). The different properties of nanoparticles and biological moiety that influences their interaction are the surface of nanoparticle, solid-liquid interface, and contact with the biological entity at the solid-liquid interface (Fig. 11.4). The most crucial attribute of the nanoparticle is the surface property which is altered by the chemical composition of material used for its synthesis and morphological properties like shape and size, porosity, hydrophobicity/hydrophilicity, presence of functional groups, crystallinity, etc.

11.6.2.1 Effect of Nanoparticle Size on the Interaction with Microbes

Nanoparticle size has been shown to influence the interaction of nanoparticle and the microbes. For example, the size of the nanoparticle has been potentiating its antibacterial activity (Slomberg et al. 2013). The greater antibacterial activity is achieved by a smaller size of the nanoparticle due to high surface area-to-mass ratio

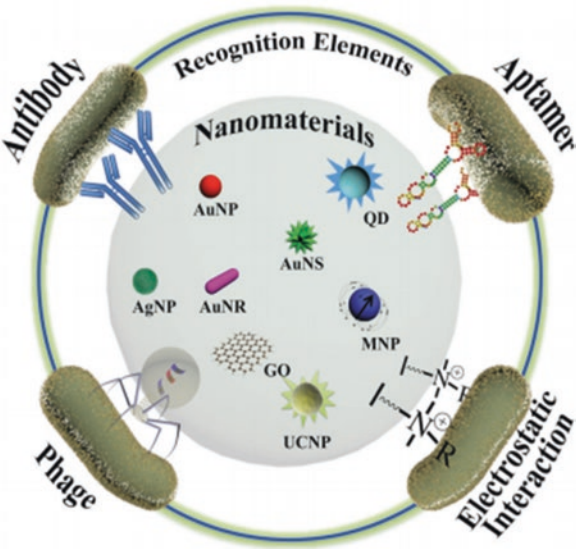

Fig. 11.4 Schematic representation of nanomaterials that can be designed as bacteria-selective nanoprobes to detect and sense bacterial cells. (Reproduced from Chen et al. (2017) by permission of The Royal Society of Chemistry)

which leads to the prevention of biofilm formation. In a study, the reduction rate of iron oxide nanoparticles by *Shewanella oneidensis* MR-1 which is an iron-reducing bacterium was found to be dependent on the size of the nanoparticles (Bose et al. 2009).

11.6.2.2 Effect of Nanoparticle Shape and Agglomeration Potential

Shape and agglomeration of nanomaterials greatly influence the cellular internalization event. The kinetics of the cellular uptake is affected to a great extent with shape and size of nanoparticle (Verma and Stellacci 2010). It is reported that spherical nanoparticles are easily uptaken than the rod-shaped cells due to the short time span for the cell membrane to wrap the spherical cells. Only few reports have explicitly studied the quantitative relationship between shape and agglomeration state with its bacterial interaction. Agglomeration has been known to affect the dissolution of a nanoparticle and its atomic structure (Arakha and Jha 2018). In the biological in vitro and in vivo studies, the nanomaterials display various attributes with various shapes and sizes which influence their toxicity toward a bacterial/animal cell. A study depicting the interaction of silver nanoparticles with *Escherichia coli* involved a comparison of different shapes of nanoparticle like spherical, rod-shaped, and triangular plate-shaped. The antibacterial activity of triangular nanoplates was found to be dominant because of being more chemically reactive and possessing high atomic density (Pal et al. 2007).

11.6.2.3 Effect of Surface Functionalization on Its Interaction

The size of the nanoparticles is commensurate with the bacterial systems; however, their interaction can be modified through appropriate functionalization approaches. Nanoparticles in addition to a core element possess many surface groups or chemical moieties that determine their physicochemical properties. The surface coating of nanoparticles can influence the hydrodynamic size, surface charge, scavenging of free radicals, and steric properties (Aruguete and Hochella 2010). Various studies have highlighted the issues of toxicity and penetration drawbacks of nanoparticles in biofilms. For example, silver-coated superparamagnetic iron oxide NPs (SPIONS) with ligands exhibited remarkable compatibility with the healthy cells and also potent antimicrobial activity. The biofilm formation was inhibited due to enhanced activity of the surface-coated nanoparticle with the cell (Mahmoudi and Serpooshan 2012). Positively charged coatings or functionalities have been known to be toxic due to their affinity to the negatively charged nanoparticles (Aruguete and Hochella 2010). The effect of surface coating will allow the elucidation of the behavior of nanoparticle in natural condition.

11.7 Conclusions and Future Perspectives

The importance of the plant microbiome for the development and functioning of plants has been widely accepted, but majority of the work done till now lacks either a quantitative assessment of the contribution of the different factors involved (host genotype, the microbiome, and environmental conditions) or a sound detection of the microbiome components. Sensing the presence of different microbes in the soil allows scope for their functional utilization in downstream studies. As such, there is a critical need for sound detection and identification of bacteria in the microbiome so that their growth promontory benefits can be maximized. Understanding the microbial interactions between different genomic elements and bacterial cells is helpful to design specific detection strategies for bacteria. Coupled with this, the proper choice of nanomaterials will allow access to and development of sensitive signal transduction methods to detect bacterial cells as “advanced biosensors.” Appropriate nanomaterials can now be utilized for detection of plant growth regulators produced by bacteria along with functionally active genes from bacterial genomes. Although some advancements have been made in this field, there remains a need to generate more sensitive systems that are able to rapidly identify bacterial species as well as differentiate them at the strain level alongside determining the beneficial biomolecules produced by them that can be applied to soil for improving agricultural productivity. This nanobionics approach to modulate and study plant-microbe interactions will lead to a new research field at the interface of nanotechnology and plant-microbe interactions. Future work will need to explore efficiency of single vs multiple species detection, and these studies will need to be combined with effectiveness of detection of bacterial beneficial molecules produced in soil for the development of competent nanosensors for microbes. In the future, more studies should be carried out utilizing innovative nanosensors that are more flexible so as to promote large-scale use in assessing soil microbes (bacteria and even fungi) as conventional diagnostic techniques are labor-intensive and require to be performed on sophisticated equipment. The ability to realize such opportunities depends on improved understanding of nanosensor behavior and trafficking within the bacteria, as well as its toxicity, specificity, and efficiency.

Future work will invariably involve the development and use of selective nanosensors to tap into the intricate microbial signaling mechanisms, providing us with previously inaccessible information about microbial responses and functions in the microbiome under different environmental conditions.

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Chapter 12
Applications of Carbon-Based Nanomaterials for Antimicrobial Photodynamic Therapy

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12.1 Introduction

The discovery of antibacterial agents is found to be the most significant development of the twentieth century in healthcare sector and has become a pioneer in current medicine in reducing the percentage of folds mortality due to bacterial diseases. In earlier periods, specifically at the time of the pre-antibiotic era, bacterial infections were found to be a major threat to humans, where the case of mortality rate for the infections caused by *Streptococcus pneumoniae* and *Staphylococcus aureus* claimed as high as 40% and 80%, respectively. Furthermore, amputation was the only option to treat the wound infection before the emergence of antibiotics; the report said that nearly 70% of amputations were exhibited during World War I as an impact of wound infections (Friedman et al. 2016). Antibiotics have facilitated the relief to the patients with bacterial infections, which drastically changed the way to treat and cure such deadly bacterial diseases. Nevertheless, antibiotics stood as supporting pillars in the advancement of modern medicine like complex surgery, transplantation, and chemotherapy. Unfortunately, the spread of bacterial pathogens with multidrug resistance to antibacterial agents found to be a major threat and significant challenge for the current treatment modalities (Sharland et al. 2015).

Development of the resistance by bacteria could be achieved either by mutation or accepting mobile genetic elements that hold resistant genes. It could occur due to the consequences of indiscriminate use of drugs which created selective pressure to the bacteria. There was a driving force behind the gradual increase in the rate of drug resistance due to the misuse of antimicrobial agents, either prescribed to the

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patients or deposited into the environment (Roca et al. 2015). Furthermore, food chain is found to be another important cause for the development of multidrug-resistant bacteria, which subsequently alters both the commensal and pathogenic bacteria in humans through dietary contact (Fig. 12.1) (Liebana et al. 2013; Tetro 2018). In a generalized view, the bacterial infections that are caused by multidrug-resistant strains contribute comparatively greater adverse effects than similar infections caused by susceptible bacterial strains. The adverse effects cover both clinical such as death or failure of treatment and economic aspects, including cost of treatment and duration of the hospital stay during the bacterial infection treatment process. The hardships of these adverse effects have been pronounced in several ways including disease severity and strain virulence (Chaudhary 2016). There was a continuous encountering of challenges in the prevention of bacterial diseases with the current situation where there is a demand for effective drug to combat the antibiotic-resistant bacterial infections. Though few new antibiotics are in the clinical pipeline, the situation seeks for the development of different novel therapeutic alternatives (Frieri et al. 2017).

In the recent years, understanding the virulence strategies and molecular identification of the disease increased significantly, which provided a novel path where virulence-associated traits of the pathogens can be targeted. This approach has been greatly appreciated due to their mechanism of targeting pathogenic factors or virulence traits rather than providing selective pressure on the pathogen to develop resistance (Hancock et al. 2012). As a result considerable efforts have been rendered to formulate drug molecules that invade the activity of the pathogenic factors which arrest pathogens until the activation of host immune system to kill the pathogens. The frequent administration of antipathogenic drugs could not show any sign of the resistance, unlike conventional antimicrobial agents, because they do not directly show adverse effects on the growth of pathogens and therefore prevent the development of resistant bacteria (Hauser et al. 2016). The bacterial toxins play a major role in the pathogenicity of the bacteria. The bacterial toxins were synthesized inside the

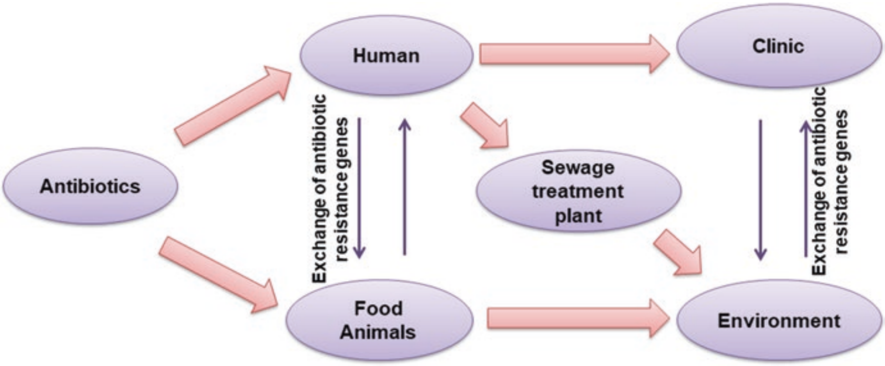

Fig. 12.1 Different modes of antibiotic resistance spread between people, animals, and the environment

cytosol, which need to cross the bacterial cell wall before they invade into the host cell. To accomplish this, the bacteria evolve with several numbers of secretion systems that facilitate the transport of the bacterial toxins to the surrounding environment. Recently, a drug has been developed to inhibit the type 3 secretion system as it is widely accepted as a potential drug target to develop anti-pathogenic drugs (Marshall and Finlay 2014). Furthermore, several leading approaches were formulated often to expand the efficacy of the antipathogenic agents because bacteria continuously alter their pathogenic mechanism; hence, the development of drug-targeting virulent traits may enhance the probability of success in the antimicrobial drug development process.

Besides the bacterial toxins, bacterial biofilm formation is a major pathogenic factor that often occurs on the inert surfaces like catheters or prosthetic joints and even in human body parts including heart valves and teeth (Khameneh et al. 2016). The biofilms are the physiological states of the bacteria found difficult to eradicate since the extracellular matrix prevents the penetration of antimicrobial agents into the biofilms (Smith 2005). The steps to eradicate this organization of cells demand significantly greater concentration of drugs for persistent periods, and these efforts have often failed due to the prolonged period of the infections. Apart from the clinical complications and treatment limitations, biofilms can also be considered as a major source of infection, which are observed in the medical apparatus (Frieri et al. 2017). The constant research in science provides a better understanding about the biofilm formation and the factors required which provide insights for the development of novel therapeutic approaches to prevent biofilm formation and also for the destruction of mature biofilms (Brooks and Brooks 2014). As the results are not promising, new strategies were reported with significant activity; however, very few of them are in the pipeline for the clinical testing (Teh et al. 2018).

Currently, an impressive approach, the inhibition of bacterial quorum sensing, has been found to be a promising alternative strategy to combat the biofilm-forming bacteria, since bacterial quorum sensing (QS) plays a significant role in the formation of biofilms (Li and Tian 2012). Moreover, the small diffusible signaling molecules in the bacterial QS participate as switching regulators that promote the planktonic bacterial cells to form biofilms. Hence, targeting these signaling molecules could be considered as a promising method to prevent infection from the biofilm-forming bacteria. Similarly, the target could be focused on the factors that facilitate bacteria in biofilms to develop resistance to antibacterial drugs, which might render a situation where biofilms can be sensitive to antibacterial drugs. In addition, other novel methods like phage therapy (Fu et al. 2010; Harper et al. 2014) and modulation of the microbiome (Biliński et al. 2016; Crow et al. 2015) have been reported as alternative approaches for the conventional antibiotics to prevent the bacterial infections and minimize the development of resistance to antibacterial drugs.

Resistant strains are developing rapidly with certain pathogenic isolates such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and members of the family *Enterobacteriaceae*, for example, *Klebsiella pneumoniae*, *E. coli*, and *Proteus* sp. (Basak et al. 2016). Among various novel antimicrobial approaches, as discussed above, antimicrobial photodynamic therapy (aPDT) has become a potential alternative against various microorganisms. Antimicrobial efficacy of photodynamic therapy was described by Oskar Raab in 1900, who observed reduction in the viability of *Paramecium caudatum* upon light exposure in the presence of acridine dye. Though Oskar Raab introduced this technique 30 years before penicillin discovery, research on antimicrobial photodynamic therapy (aPDT) was limited after the discovery of antibiotics. However, currently, due to the rapid increase in the multidrug-resistant strains, aPDT is extensively studied and used in the treatment of localized infections (Oruba et al. 2015).

12.2 Antimicrobial Photodynamic Therapy

Photodynamic therapy involves the use of mainly three components to kill undesired microorganisms: a nontoxic light-sensitive compound (photosensitizer), a visible light source, and oxygen (Wong et al. 2005). aPDT is an oxygen-dependent photochemical reaction that occurs upon light-mediated activation of a photosensitizer compound which leads to the generation of cytotoxic reactive oxygen species or singlet oxygen (Garcez et al. 2011). On exposure to light of a specific wavelength, the photosensitizer absorbs energy and undergoes transition from a low-energy ground state to an excited singlet state. In this excited singlet state, the boosted electron in the higher energy orbital still maintains its opposite spin. But this state is short-lived (nanoseconds) and subsequently loses its energy by fluorescence or internal heat conversion. The excited singlet-state PS can also invert its electron to parallel spins by a process called intersystem crossing. The change of electron to parallel spins results in the formation of an excited triplet-state PS which is a long-lived state (microseconds). The triplet-state PS can transfer its energy in two different pathways: type I and type II photoprocesses, both of which are oxygen dependent (Robertson et al. 2009). Type I reaction involves electron transfer from the excited triplet-state PS to organic substrates of the cell resulting in the production of free radicals which are highly reactive and react with molecular oxygen to generate reactive oxygen species (ROS) like superoxide, hydroxyl radicals, and hydrogen peroxide. These reactive oxygen species are cytotoxic in nature which cause damage to cell membrane integrity and permeability. In type II pathway, the excited triplet-state PS transfers its electron to a ground-state molecular oxygen (triplet state) that turns into a highly reactive singlet oxygen. This species oxidizes many compounds like nucleic acid, proteins, and lipids leading to oxidative damage of the cell wall (Hamblin and Hasan 2004). Both the mechanisms target mainly biomolecules like amino acids, unsaturated lipids, and purine and pyrimidine bases of nucleic acids (DNA/RNA). Since aPDT targets a wide range of biomolecules at a time, it is considered as a broad-spectrum antimicrobial therapy. This broad-spectrum activity gives advantage to aPDT over antibiotics as it is difficult for microbes to gain resistance against wide targets of aPDT at a time (Sperandio et al. 2013).

12.2.1 Mechanisms of aPDT

Photodynamic therapy is a nonthermal photochemical reaction based on the excitation of a photosensitizer upon exposure to visible light of a particular wavelength. Delivery of light with appropriate wavelength to a PS leads to absorption of photon resulting in the transformation of PS to an excited singlet state (^{1}PS) from ground state (^{0}PS). The reason behind this excitation is electron transfer from the highest occupied molecular orbital to one of the unoccupied molecular orbital. This state is called the excited singlet state where an opposite electronic spin is maintained as in the ground state. The excited singlet state is a short-lived state (nanoseconds), and the molecule can revert back by releasing the absorbed energy via fluorescence or by interconversion where energy lost is in the form of heat (Oruba et al. 2015). From the excited singlet state, it can change to the excited triplet state by spin conversion where it forms a parallel spin (intersystem crossing). The excited triplet state has lower energy than the singlet state but is stable and long-lived (micro to milliseconds). This triplet-state molecule can also release energy and revert back to the ground state through phosphorescence or through a nonradiative pathway. There is also another mechanism by which it can transfer its energy to the surrounding molecules (Fig. 12.2). Based on the molecules to which they transfer energy, there are two different types of PDT—type 1 and type 2 (Sudhakara et al. 2012).

Type 1 reaction involves generation of superoxide and hydroxyl radicals. The excited triplet-state molecule transfers its electron to the surrounding substrate molecules in the cell and produces radical anions or radical cations which may further react with the oxygen molecule to produce ROS. ROS damage the cell through the formation of oxidative stress. Type 2 pathway is based on the production of highly reactive and toxic singlet oxygen ($^1O_2$) through direct transfer of energy from the

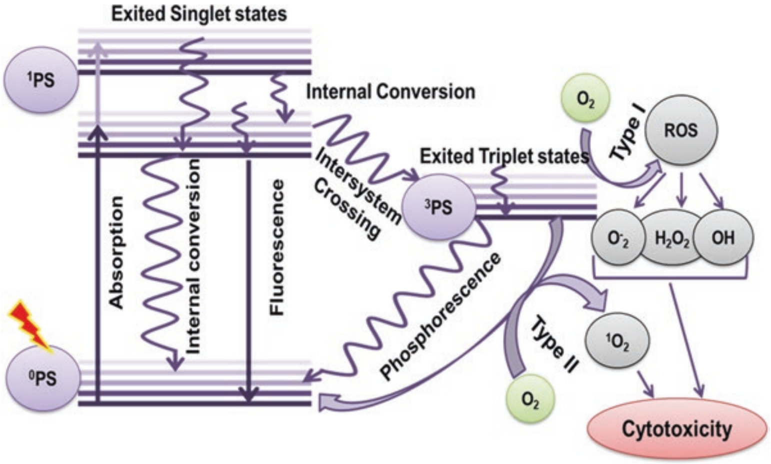

Fig. 12.2 Schematic illustration of mechanism involved in antimicrobial photodynamic therapy

triplet-state molecule to ground-state oxygen molecule (in triplet state) thereby converting it to reactive toxic singlet-state oxygen (Spagnul et al. 2015). All these ROS generated have the ability to target multiple sites at a time. They kill cells by damaging nearly all types of biomolecules (nucleic acid, protein, and lipids) which prevent microorganisms from developing resistance against aPDT making it as a promising and successful alternative to antibiotics (Hamblin 2016).

12.2.2 Recent Scope of aPDT

The recent research on aPDT is focused on multidrug-resistant pathogenic bacteria which readily form biofilms and cause dental caries, chronic wound, and skin infections. The efficacy of antimicrobial and antibiofilm effects of PDT on *Streptococcus mutans*, a cariogenic bacterium, playing an important role in dental caries was studied. The study was carried out with toluidine blue as a PS and diode laser as a light source, resulting in a significant reduction of biofilm formation (63.87%) compared to control. This can be used as an alternative methodology for treating oral diseases which is comparatively advantageous to chemical or mechanical or antiseptic agents as these methods would cause inactivation of normal microbiota and mechanical damage to oral mucosa and promote development of drug-resistant strains (Beytollahi et al. 2017). One of the most important pathogen causing clinically relevant infections within immunocompetent patients is *S. aureus*. Several studies of aPDT were performed to inactivate biofilm producers, both *S. aureus* and MRSA. An effective disruption of *S. aureus* biofilm in compact and cancellous bones (in vitro) by aPDT using 660 nm diode laser with methylene blue dye was reported. Results indicated a 10 log reduction of *S. aureus* biofilm in compact and cancellous bones compared to control group (Rosa et al. 2015).

Application of aPDT was also studied in the case of prosthetic joint infection (PJI) caused by the biofilms of MRSA and *P. aeruginosa* on prosthetic implants. PJI has become a public health concern, and due to the biofilm formation by multidrug-resistant strains, a broad-spectrum nonantibiotic antimicrobial treatment is required which is fulfilled using aPDT. The therapy was conducted with the help of a novel PS RLP068/Cl under diode laser exposure. A significant decrease in biomass volume of both *P. aeruginosa* and MRSA strains was observed, indicating the successful antimicrobial and antibiofilm activity of aPDT with RLP068/Cl compared to antibiotic treatment (Vassena et al. 2014). Studies on aPDT have been reported using natural PS like curcumin which has proven to be nontoxic to a number of cell cultures and animal tissues. But the phototoxicity of curcumin is well-known and can be used as a PS in aPDT to treat localized superficial infections on skin and mouth. Biofilms of cariogenic bacteria like *S. mutans* and *L. acidophilus* were subjected to aPDT, and their enhanced biofilm disruption due to phototoxic effect of curcumin under a light-emitting diode (LED) of 450 nm was analyzed (Araújo et al. 2012).

12.2.3 Challenges in aPDT

Several studies on aPDT in vitro have reported the efficient killing of bacterial cells by the combination of PS and an appropriate wavelength of light. However, many of the potent PSs are having limitations such as low solubility in aqueous medium and tendency to aggregate. A decreased solubility results in a reduced uptake of dye by cells, i.e., bioavailability and aggregation effect efficiency of PDT (Hegge et al. 2010). Moreover, long-term storage and light exposure can also lead to degradation of PSs. Hence, to enhance the permeability and retention effect of PS, there should be a vehicle to carry PS into an appropriate target. The effectiveness of PDT depends on many factors like the degree of ROS production, concentration of PS, targeted delivery, and uptake (Rout et al. 2016).

12.3 Nanomaterial in aPDT

Although the mentioned methods are found to be the novel and alternative ways to eradicate the bacterial infection, still there is a possibility that bacteria could be able to establish a resistance mechanism to these methods. Because bacterial pathogens are evolving at a greater speed, this facilitates the bacterial species to gain resistance against antibacterial drugs by several mechanisms including modification in membrane permeability, development of the multidrug efflux pumps, degradation of the drug by enzyme, self-mutation, and covalent alteration of antibiotic molecules which result in the inactivation of antimicrobial properties (Pelgrift and Friedman 2013). These factors make the situation much complicated, indirectly boosting the resistance mechanism in pathogenic bacteria. The recent development in nanobiotechnology mediates the current research to focus on these complications to provide a proper solution for these problems, potentially elevating the efficacy of the available antimicrobial drugs by using nanoparticles as the delivery system to achieve targeted therapeutic practice (Zhu et al. 2014). In the last decade, nanobiotechnology has arrived at a magnificent growth that provides several applications in the healthcare sector. Nanomaterials are observed as multitasking agents in disease management programs, where they could appear as promising antimicrobial agents, predominantly against multidrug-resistant bacteria (Table 12.1). Furthermore, the nanomaterials were extensively used as drug carriers that could deliver the traditional antibiotics to the precise site, which promote the target-specific therapeutic approach. In addition to the several applications of nanoparticles, they are also used as promising tools in the diagnosis of infectious diseases. Currently, several nanosystem-based diagnostic tools are available to precisely detect the infectious organisms at the early stage of the infection. For example, nano-based diagnostic tools are developed to specifically detect disease-causing agents or to distinguish Gram-positive and Gram-negative microorganisms. As an advancement of this approach, a nano-ranged diagnostic tool has been developed and applied in vital

Table 12.1 List of different carbon nanomaterials and their antimicrobial efficacy

S.no.	Carbon nanomaterial	Size	Application	Targeted microorganism	Activity	References
1.	Fullerenes	10–25 nm	Antibacterial agent	*Bacillus subtilis*	Antibacterial activity	Lyon et al. (2006)
		2–200 nm	Antibacterial agent	*Escherichia coli*	Antibacterial activity	Deryabin et al. (2014)
		122–295 nm	Antibacterial agent and nanocarrier	Bacteriophage	Antibacterial and antiviral	Dostalova et al. (2016)
2.	Graphene and graphene oxide	100–200 nm	Antibacterial agent	*E. coli*, *Staphylococcus aureus*, *Enterococcus faecium*, and *Klebsiella pneumoniae*	Antibacterial activity	Whitehead et al. (2017)
		24–4923 nm	Antibacterial agent	*E. coli* and *S. aureus*	Antibacterial activity	Gao et al. (2017)
		–	Antibacterial agent	*E. coli*	Antibacterial activity	Sharma et al. (2018)
3.	Carbon nanotubes	1–5 μm (length)	Antibacterial agent	*Salmonella enterica*, *E. coli*, and *E. faecium*	Antibacterial activity	Dong et al. (2012)
		–	Antimicrobial agent	*S. aureus*, *E. coli*, and *Candida tropicalis*	Antimicrobial activity	Venkatesan et al. (2014)
		40–60 nm (diameter) 5–15 μm (length)	Antibacterial agent	*Methylobacterium* spp.	Antibacterial activity	Choi et al. (2014)
4.	Carbon quantum dots	5–6.5 nm	Antibacterial agent	*E. coli* and *B. subtilis*	Antibacterial activity	Travlou et al. (2018)
		5 nm	Antibacterial agent	*S. aureus*, *B. subtilis*, and *E. coli*	Antibacterial activity	Li et al. (2018)
		10 nm	Antibacterial agent	*S. aureus* and *E. coli*	Antibacterial activity	Roy et al. (2015)

detection, which, along with the available analytical technology, is gaining significant attention due to its superiority (Colino et al. 2018). The overall explanation summarize, though nanoparticles contributing several applications, two of them are highly consider as promising including application of inorganic and organic nanomaterial as potential antimicrobial agents and nanomaterial based drug delivery system. The nanomaterials like metallic nanoparticles, carbon nanotubes, fullerenes, cationic peptides, or polymeric nanoparticles and chitosan nanoparticles were being considered that they were inherent with antimicrobial properties and used widely in antimicrobial applications. On the other hand, the nanomaterials including liposomes, solid lipid nanoparticles, polymeric nanoparticles, silica nanoparticles, and gold nanoparticles are extensively employed as potential drug carrier systems (Zaidi et al. 2017; Prasad et al. 2017).

Among the enlisted nanomaterials, carbon-based nanomaterials have gained a lot of attention due to their exceptional structural characteristics, physical properties, tunable morphologies, relatively good biocompatibility, and eco-friendly nature. The emergence of functionalized carbon-based nanoparticles provided a new class of nanocarriers for the effective delivery of drug molecules to the appropriate sites of infections (Ibrahim et al. 2018). The application of these nanomaterials as a delivery system is widely accepted as a promising approach, since they potentially mobilize the therapeutic molecules into the bacterial cytoplasm (Arias and Yang 2009). Interestingly, these nanocarriers could be formulated with one or more bioactive molecules like therapeutic compounds, antimicrobial peptides, biosensing proteins, and nucleic acid which subsequently render the antimicrobial agents to the cells or organs (Shvedova et al. 2008). The surface functionalization on the carbon nanomaterial prevents the attachment of unwanted absorption and desorption processes. The unwanted absorption that is mentioned in this case is the adherence of substances from the biological system to the carbon nanomaterial at the time of drug administration which would minimize the original effect of the nanoformulated drugs and have greater impact on their release profile and target specificity (Mocan et al. 2017). Comparing to other nano-based therapeutic approaches, carbon-based nanoparticles are found to be more effective and cost-effective. For example, the preparation of carbon nanomaterial with antibiotics is less costly than the preparation of the nanomaterial, liposome, with antibiotics. Additionally, in both in vitro and in vivo studies, the antibiotic that was administered along with the carbon nanocarrier was found to be more effective than the free antibiotic (Prajapati et al. 2011).

It is apparent that carbon is one of the most abundant elements on the Earth's crust, and it possesses the ability to couple with other carbons to differentiate into several allotropes of carbon. This characteristic feature of carbon provides the possibility to drive several varieties of carbon nanomaterials that include fullerene, nanodiamonds, carbon dots, graphene quantum dots, and carbon nanotubes (Maas 2016). The nature of the carbon nanomaterial was altered by different hybridization states of carbon, for example, carbon nanotube, fullerene, and graphene are made with sp^2-hybridized carbon atoms. Similarly, nanodiamonds are composed of sp^3-hybridized carbon atoms; likewise, carbon dots and graphene quantum dots have mixed sp^2- and sp^3-hybridized carbon along with defects and heteroatoms. Though the orbital hybridization of all the carbon nanomaterials was found to be more likely similar due to their change in the dimensions, each carbon nanomaterial showed difference in their antimicrobial activity and mechanism of action (Xin et al. 2018). Interestingly, the similar dimensional carbon nanomaterials show change in the antimicrobial activity when their other parameters such as size, shape, number of layers, charge of the particles, and the surface material on the carbon nanomaterial have been altered. The specific physicochemical properties of the carbon nanomaterial could be mainly dependent on the method that is followed for the preparation of that particular carbon nanomaterial (Maleki Dizaj et al. 2015).

12.4 Carbon-Based Nanomaterial in aPDT

Due to the development of antimicrobial-resistant strains over the last decades, the need to design novel and efficient antimicrobial agents resulted with the integration of nanotechnology. The rise in the drug resistance among microorganisms has led to the discovery of potential and alternative therapeutic strategies including antimicrobial photodynamic therapy (aPDT). As discussed above, aPDT can be enhanced by the use of nanoparticles either as an antimicrobial agent or for the effective delivery of the photosensitizer (Perni et al. 2011). Among the different groups of nanoparticles available, carbon-based nanomaterials are of great interest and effective in aPDT. The efficient and potential use of carbon nanomedicine in aPDT is attributed to their excellent optical properties, good biocompatibility, less toxicity, and excellent mechanical strength. These carbon nanomaterials and their derivatives gained significant interest in several fields due to their excellent physical and chemical properties. They are widely used in thin-film transistors, photovoltaics, electrodes, supercapacitors, drug delivery, tissue engineering, and photothermal therapy (Al-jumaili et al. 2017).

Carbon nanomedicine is an emerging field which combines antimicrobial photodynamic therapy for the treatment of notorious pathogens. These nanoparticles are employed in PDT either as photosensitizers or drug vehicles. The ability of the carbon-based nanomaterials to carry photosensitizers or to exert antimicrobial action can be enhanced by the functionalization methods. Covalent and noncovalent modes of functionalization strategies are available which render the efficient application of carbon in nanomedicine (Albert and Hsu 2016). The covalent functionalization strategies include the conjugation of polymers to NPs using polyethylene glycol and hydrophobic functional groups using carboxyl and hydroxyl groups. The covalent functionalization also targets ligands for effective conjugation with carbon-based nanoparticles. Weaker interactions such as van der Waals forces, hydrophilic interactions, hydrophobic interactions, electrostatic forces, and pi–pi stacking are the noncovalent functionalization strategies. Among these methods, covalent functionalization produces more stable carbonaceous nanoparticles due to their ability to form covalent linkages between NPs and biomolecules (Chen et al. 2012; Zhu and Xu 2010). Carbonaceous nanomaterials such as carbon nanotubes, fullerenes, graphene oxide, carbon nanodots, etc. are widely used as photosensitizers or employed to improve the delivery of PS in aPDT (Fig. 12.3).

12.4.1 Carbon-Based Nanomaterials and Their Properties

Carbon-based nanomaterials exhibit high microbicidal activity along with their photosensitizing capacity. The antimicrobial properties of carbon-based nanoparticles are dependent on their small size and high surface-to-volume ratio with an excellent interaction with bacterial pathogens. As the size and surface area of

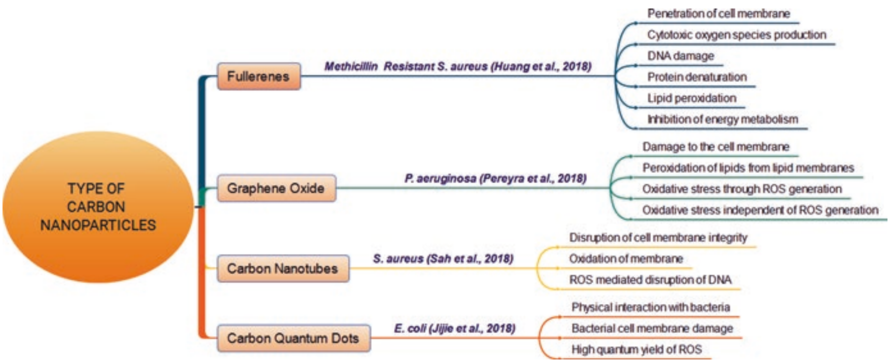

Fig. 12.3 Different types of carbon nanostructures employed in antimicrobial photodynamic inactivation

nanoparticles are the important parameters affecting microbicidal action, other factors such as the type of target microorganisms, functionalization yielding surface modification, intrinsic properties, and composition of carbon-based nanomaterials also matter (Buzea et al. 2007; Kang et al. 2008). The death of bacteria is mainly due to the physical interaction of bacteria rather than the phototoxicity. The carbon-based nanomaterials which make contact with the bacterial membrane cause the production of free radicals and lead to the oxidative death of cells. The oxidative stress of bacteria is enhanced by the use of photoactivated carbon nanoparticles (Manke et al. 2013; Abrahamse et al. 2017). Briefly, the antimicrobial action of carbon-based nanoparticles can be grouped as physical and chemical mechanisms. The interaction with microorganism and significant structural damage are known to be the physical mechanisms of bactericidal action. Chemical interaction leads to the production of reactive oxygen species or cause an ROS-independent cell death due to the electron transfer from the outer surface of the microorganism (Li et al. 2015). Other advantages of carbonaceous nanomaterials in aPDT include easy design, synthesis, fast elimination, less cytotoxicity, and good penetration (Abrahamse et al. 2017).

12.4.2 Fullerenes

Researchers are engaged in the design of new and efficient photosensitizers in order to enhance the efficacy of aPDT. Fullerenes came into field due to their high drug carrier or loading capacity. Fullerenes have a unique structure with biologically inert molecules of 60–100 carbons arranged in a soccer ball shape. The fullerenes are characterized by the extended and conjugated molecular orbitals (Constantin et al. 2010). Fullerenes have huge applications in nanoelectronics, nanocomposites, and drug delivery because of their tensile strength, high electroconductivity, and unique optical and thermal properties. C_{60} and C_{70} fullerenes are insoluble in water and made soluble by preparing their derivatives. These fullerene derivatives especially of C_{60} have shown strong antibacterial effects on prokaryotes through the direct oxidation of cells (Li et al. 2008). The antimicrobial properties of nano-C_{60} fullerenes were studied previously on laboratory bacteria, *E. coli* and *B. subtilis* (Yacoby and Benhar 2008). Nano-C_{60} has UV/visible absorption properties in solutions which cause the generation of free radicals. Some of the fullerenes showed the ability to cause free radical damage to bacterial cells. Free radicals-induced damage to bacteria was marked by lipid peroxidation and DNA damage (Nakanishi et al. 2002).

One of the fullerene derivatives, fullerol, showed higher phototoxicity toward MS2 bacteriophage than other nanoparticles. MS2 bacteriophage was efficiently inactivated by UVA light-activated fullerol (Badireddy et al. 2007). In a study, it was showed that carbonaceous nanoparticles exhibited more photoinactivation than TiO_2 as they produce more singlet oxygen (Brunet et al. 2008). Fullerenes show a large absorption in the visible light and good quantum yield in the triplet electronic states. Different functionalizations using hydrophilic and amphiphilic groups or fused ring structures render the fullerenes with water solubility and protect from aggregation. The attachment of groups which provide them cationic charges offers several roles of broad-spectrum bacterial targeting and water solubility. This also imparts them with the ability to produce higher amounts of free radicals such as superoxide anion, hydroxyl radicals, and singlet oxygen for effective aPDT. Fullerenes have recently gained interest in the research of photodynamic treatment for infections as they possess higher degree of photostability compared to traditional tetrapyrrole ring-based PSs (Mroz et al. 2007). Generally the absorption range of fullerenes is in the range from UVA (360 nm) to red (635 nm), which makes them effective candidates for aPDT (Mizuno et al. 2011; Sharma et al. 2011). Yin et al. (2015) investigated the aPDT of broad-spectrum microbial strains using new decacationic fullerene derivatives such as $C_{60}[{}_4M(C_3N6\text{þ}C_3)_2]–(I)_{10}$(LC14), $C_{60}[{}_4CPAF–(MN_6\text{þ}C_3)_2]–(I)_{10}$ (LC15), and L16 (malonate bisadduct derived from C14), illuminated with white light and UV light. They showed that the LC15 derivative was the most powerful broad-spectrum antimicrobial PS followed by LC16. LC14 was found to be with the least antimicrobial action. The molecular mechanism behind the enhanced ROS production was through type 1 electron transfer method which increased the ability of monoadduct fullerene derivative to kill microorganisms (Yin et al. 2015).

The mechanism of fullerenes and their derivatives under irradiation includes the penetration of cell membranes and destruction of nucleic acids, proteins, and lipids resulting in strong antibacterial, antiviral, and antioxidant activities. The photodynamic inactivations of viruses using photoactivated fullerenes are widely studied. Rud et al. (2012) suggested the use of photoactivated C_{60} fullerenes for the photoinactivation of mosquito-iridescent virus (MIV) *Aedes flavescens* under 30 min of illumination in biological systems. The photoexcited C_{60} reduced the infectious viruses by 4.5 lg ID_{50}/mL units (Rud et al. 2012).

Photoactivated fullerenes, functionalized with three dimethyl pyrrolidinium groups (BF6) using white light, cured fatal infections caused by *Proteus mirabilis* and *Pseudomonas aeruginosa* in mouse wound models (Lu et al. 2010). Recently, water-borne bacteria *Enterococcus faecalis* was photoinactivated by C_{60} and its derivative immobilized onto a macroporous silicone (a support polymer) (Manjón et al. 2014). Aoshima et al. (2009) used water-soluble fullerenes encapsulated into different carriers and three types of fullerenols for the photoinactivation of *Propionibacterium acnes*, *Staphylococcus epidermidis*, *C. albicans*, and *Malassezia furfur*. In the study fullerenols showed stronger activity against fungi than bacteria which further explains that water-soluble nanoparticles could interact more with the fungal cell wall components (β-glucan and chitin) than the peptidoglycan layer of the bacterial membrane (Aoshima et al. 2009). The possible targets of fullerenes include cell membrane, lipids, and DNA. The damage to the biological constituents is either caused by type I or type II mechanism. Recent reports are available with the effective broad-spectrum antimicrobial photodynamic therapy using fullerenes functionalized with methylpyrrolidinium groups. Grinholc et al. (2015) employed C_{60} functionalized with methylpyrrolidinium groups to kill Gram-negative, Gram-positive, and fungal cells following the irradiation with white light (Grinholc et al. 2015).

12.4.3 Graphene and Graphene Oxide (GO)

Graphene is generally called as the simplest structure among all carbon nanomaterials and possesses structural similarities with the 3D closed cage of fullerenes, 3D rolled tube of carbon nanotubes, and 3D stack sheets of graphite. Graphene is structurally characterized as a 2D sheet of carbon atoms with sp^2 and sp^3 hybridization. Graphene oxide has a 2D honeycomb lattice structure (Wang et al. 2011). Graphene is known to be the simplest form and thin material of carbon so far. Few layers of graphite are arranged in the closely packed honeycomb structure. The different members of graphene family include reduced graphene oxide, graphene oxide, graphene sheets, few-layered graphene, and multilayered graphene (Priyadarsini et al. 2018).

Many scientists have been attracted toward GO because of its water solubility, chemical inertness, optical transmittance, density, biocompatibility, large surface area, and stability in aqueous solutions. The large specific surface area of GO has enhanced its ability to carry and deliver hydrophobic drugs such as doxorubicin. This is because of its ability to solubilize hydrophobic moieties between the graphene sheets due to the pi–pi stacking (Sun et al. 2008; Huang et al. 2012). Graphene is known as the rising star of carbon nanomaterials due to its superior properties. Hence, it is widely studied in near-infrared photoinactivation of broad-spectrum microorganisms (Deokar et al. 2017). Mesquita et al. (2018) reviewed the improved delivery of drugs through nanographene oxide for root canal disinfection. The study showed more antimicrobial (2.81 log) and antibiofilm (94%) activities against *E. faecalis* than the free drug under illumination (Mesquita et al. 2018). Gholibegloo et al. found that multifunctionalized graphene oxide could be used as a nanocarrier for efficient loading of indocyanine green and enhanced antibacterial activity against *S. mutants* for the treatment of local dental infections (Gholibegloo et al. 2017).

Graphene quantum dots (GQDs) are synthesized with special physicochemical features of graphene. The photoinactivation ability of GQDs is mediated by the production of ROS through energy or electron-transfer mechanism upon photoexcitation which results in microbial death (Ristic et al. 2014). In a study, GQDs were found to be toxic to MRSA and *E. coli* when photoactivated with a green laser but nontoxic to mouse spleen cells indicating their specific antibacterial photodynamic inactivation property (Ristic et al. 2014). Another advantage of using graphene-based nanomaterials for aPDT is their NIR (700–1100 nm) absorption. This allows targeted and deeper cell penetration of drugs for effective photothermal therapy (Weissleder 2001). Akhavan and Ghaderi (2009) described chemically stable reduced graphene oxide/TiO_2 thin films for the photoinactivation of *E. coli* under 4 h of solar light irradiation, and it resulted in an improved antibacterial activity by a factor of 7.5 (Akhavan and Ghaderi 2009). Graphene oxide with a high loading and release of antibiotics is utilized for near-infrared photothermal therapy of pathogens. Altinbasak et al. (2018) disclosed that reduced graphene oxide integrated with polyacrylic acid with high drug loading controlled and released antibiotics on demand for the photoinactivation of pathogens. The nanofiber platforms loaded with different antibiotics were found to be potential candidates for medical applications with excellent wound-healing activity in mice infected with *S. aureus* (Altinbasak et al. 2018).

12.4.4 Carbon Nanotubes

The minuscule of CNT is reported to be significant for many biomedical applications due to their unique physicochemical properties. CNTs were categorized as single-walled and multiwalled carbon nanotubes based on the number of graphene sheets. CNTs have either open or closed ends with sp^2-hybridized carbon atom sheets. Carbon nanotubes have an axial symmetry and tubular shape with a nanoscale diameter which make them effective for the targeted release of drugs (Albert and Hsu 2016). Carbon nanotubes (CNT) may open new windows for eliminating the biomedical problems related to the bacterial virulence and infections. The unique properties of CNTs make them toxic to microorganisms. The antibacterial activity of CNTs has been widely used in the production of bacterial filters. The high binding affinity and spontaneous internalization of CNTs by bacteria have exploited them for gene/drug delivery. Further, the absorption of NIR radiation by CNTs suggested their application as efficient photosensitizers in aPDT (Kim et al. 2007). The functionalization of CNTs with various molecules can enhance the biocompatibility and cellular uptake. The conjugation or encapsulation of CNTs with other photosensitizers is a potential tool for PDT toward infectious diseases.

As CNTs are insoluble in aqueous solutions, various surface modifications especially noncovalent modifications are employed widely to maintain their original properties and improved biological applications. The enhanced interaction between

CNT and bacteria is attributed to their high surface–volume ratio and thereby decreased size. Single-walled carbon nanotubes (SWCNTs) showed more phototoxicity toward bacteria than multiwalled carbon nanotubes (MWCNTs) indicating that their nanodiameter and nanosize are two important factors in PDT treatment. The interactions between the bacterial cell and CNT result in membrane perturbation and eventually bacterial death due to the increased ROS generation (Chen et al. 2013). The mechanism of damage of microbial cells when they come in contact with the CNT is well explained by the quantum yield of reactive oxygen species. Among SWCNTs and MWCNTs, the former is found to be producing high quantum yield of singlet oxygen than the other through an energy-transfer mechanism (Aboofazeli et al. 2011).

There are several studies with regard to the antimicroial activity of CNTs. In a study, CNT scaffolds inhibited cell cycle division and reduced the cell number of *Tritrichomonas foetus*, a eukaryotic parasite after PDT. The study concluded that the production of ROS caused programmed cell death in protozoa due to the release of lysosomal protease which activates procaspases (Machado et al. 2014). Photoactivated MWCNT/TiO_2 was used for the removal of enterohemorrhagic *E. coli* (EHEC) (Oza et al. 2013). The mechanism behind the pathogen removal was explained by the generation of free radicals such as superoxide radicals and hydroxyl radicals. Visible light-activated porphyrin–MWCNT conjugates were found to be efficient antiviral therapeutics for the inactivation of influenza A virus which infects mammalian cells (Banerjee et al. 2012). SWCNT–lysozyme coatings prepared by a layer-by-layer assembly showed strong antibacterial activity against *S. aureus* and *Micrococcus lysodeikticus* (Nepal et al. 2008). Both the single-walled and multiwalled carbon nanotubes are efficient drug delivery agents. These two CNTs exhibited excellent antibacterial activity as they serve as NIR-clustering photothermal agents. Carboxyl-functionalized mutiwalled carbon nanotubes were used as a carrier of rose bengal for the effective photodynamic inactivation of *E. coli* with green laser exposure (Vt et al. 2018). Phototoxicity of CNT/agar composites was higher against *S. mutants* under NIR irradiation than graphite/agar and activated carbon/agar composites (Akasaka et al. 2010). In the study, denaturation of bacterial proteins was the mechanism of death after PDT treatment but not targeting of the cell wall.

Conjugation of PS with CNT causes an increased bacterial uptake of PS, reduced efflux of PS, enhanced metabolic intervention, and altered delivery of drugs with controlled release. Photoinduced porphyrin–SWCNT conjugates were proposed for the visible-light inactivation of drug-resistant pathogen, *S. aureus*. The interactions between the conjugate and cell wall result in the membrane damage and can be used as a potential antibacterial agent (Sah et al. 2018).

12.4.5 Carbon Quantum Dots

The extended use of semiconductor-based quantum dots has limited their application in biomedicine due to their heavy metal release and cytotoxicity. Surface functionalization of quantum dots resulted in the development of new nanocarbons such as graphene quantum dots (GQDs) and carbon quantum dots (CQDs). CQDs are very small carbon nanomaterials with several surface passivation schemes. Among the surface passivation schemes, the numbers of organic molecules used to chemically functionalize the carbon quantum dots are most effective. CQDs have special structures of core shell nanodot structures and thin shell of soft materials (Al Awak et al. 2017). These quantum dots especially carbon-based QDs have gained much interest in biomedicine due to their high solubility, low toxicity, good compatibility, modification, strong photoluminescence, and chemical inertness. The wide applications of CQDs in biomedicine make them as a rising star in the field (Y.-P. Sun et al. 2006). The surface defects of CQDs affect the yield of ROS which can be eliminated by functionalization with the amine, carboxyl, and carbonyl groups. Along with the high yield of ROS, functionalization makes CQDs more water-soluble and prevents the carbon particle aggregation (Liu et al. 2007).

Researchers showed a high quantum yield of 27% from 7.5% after the functionalization of CQD with 2, 20-(ethylenedioxy)bis(ethylamine). These photoactivated EDA-CQDs were observed with high antibacterial activity against *E. coli* and *B. subtilis* under varying conditions of treatment (Al Awak et al. 2017). Sattarahmady et al. (2017) suggested photo-ablation strategy as a minimally invasive method to fight with pathogens using carbon dots. The authors showed the reduction in the viability of wild and MRSA upon irradiation in the near-infrared range. The main reasons for the bactericidal effect exerted by CQDs were due to the ROS production and the subsequent cell wall disruption which interferes with the structure and functions of proteins, enzymes, and lipids (Sattarahmady et al. 2017). Multifunctionalized CQDs were prepared as antimicrobial agents and/or as carriers of ampicillin for effective killing of *E. coli* under visible-light irradiation. The enhanced antibacterial activity was observed due to the improved stability of antibiotic and high amount of ROS production. The ability of CQDs to attach with the bacterial cell wall exposes the bacteria to more drugs and ROS disrupting the integrity of the cell membrane (Jijie et al. 2018).

12.5 Application and Significance of Carbon Nanomaterials in aPDT

There are several studies regarding the effective and enhanced antimicrobial and antibiofilm activities of carbon nanostructures. The size and diameter of nanoparticles in nanodimensions are the two main characters determining the efficacy of aPDT. Fullerenes, single-walled carbon nanotubes, and graphene oxide were reported as potential antibacterial agents. The promising mechanism of action of these nanomaterials includes inhibition of bacterial growth, harming the electron transport chain, interruption of the energy metabolism, disruption of the cell membrane, and development of bacterial cell–nano aggregates (Maleki Dizaj et al. 2015) (Fig. 12.4). The sole properties of carbonaceous nanomaterials such as their lack of

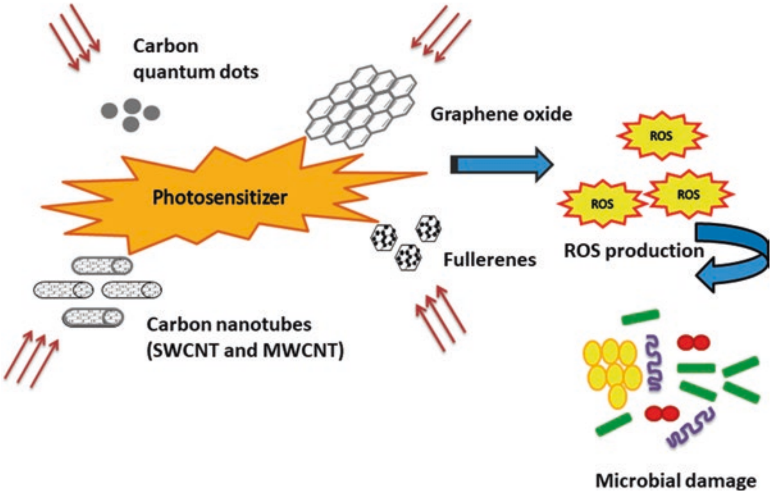

Fig. 12.4 Antimicrobial photodynamic inactivation and mechanism of action of carbon nanostructures causing phototoxicity

toxicity, good biocompatibility, large surface area, tunable morphology, water solubility, photothermal activity, and absorbance at near-infrared range make them effective candidates as photosensitizers or PS carrier molecules. Among these, the ability of carbon nanotubes to photogenerate ROS has attracted their applications toward antimicrobial therapy against infectious diseases (Mesquita et al. 2018). Carbon nanostructures are developed in a highly purified manner and functionalized with different groups for their successful application in biology and medicine. Carbon nanostructures showing an enhanced solubility in physiological solutions are considered as an encouraged methodology for the photothermal therapy of infectious diseases.

12.6 Future Perspectives and Conclusions

This chapter is mainly focused to provide scientific developments on the type of carbon nanomaterials and their application as antimicrobial and antibiofilm agents. Carbon in nanomedicine has been widely accepted by the scientific world, but the clinical application is yet to be validated. Tunable morphologies of carbon nanomaterials help to overcome the hindrance to access biological systems due to their insoluble nature. The intrinsic ability to photogenerate different types of cytotoxic species allows the integration of nanostructures into PDT as photosensitizers or carriers. Recently, carbon nanomaterials found a unique place in the ablation of cancer. Scientists expect the aPDT using carbon nanomaterials will be replaced with other modalities and theranostics for the treatment of infections and other forthcoming diseases. Contemporary evolution in carbon nanomedicine opens extraordinary opportunities for the development of new biomaterials. In particular, the proposed mechanisms of action of these carbon nanomaterials in aPDT are defined as damage to the cell membrane, separation of cytoplasm, ROS generation, and oxidative death. The antimicrobial effect is not purely dependent on the type of nanostructures, but various parameters such as light, temperature, functionalization, size, shape, electronic structure, and the nature of pathogens also influence the therapy. In general, carbon nanostructures are potential antimicrobial candidates with several biological applications because of their capacity to kill and prevent the adhesion and biofilm formation of pathogenic bacteria. The story of carbon nanostructures for antimicrobial photodynamic therapy is not fully unfolded and further research is required in this direction. Research on the exploitation of carbon nanostructures for effective photodynamic therapy against different groups of microorganisms with antibiotic resistance and their clinical application in medicine is the need of the hour. Carbon nanostructures as photosensitizers as well as reliable carriers of photosensitizers in photodynamic therapy to prevent the growth of antibiotic-resistant microorganisms and their biofilms can be considered as an effective alternative to the conventional antibiotics.

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Chapter 13
Application of Nanotechnology in Plant Protection by Phytopathogens: Present and Future Prospects

Fouad Mokrini and Rachid Bouharroud

13.1 Introduction

Crop losses due to plant disease and pests are a major threat to food security worldwide (Savary and Willocquet 2014). Plant diseases are caused by viruses, bacteria, insects, bacteria, and nematodes. Worldwide, insect pests and plant diseases are responsible for losses ranging between 14% and 13%, respectively (Agrios 2005). Recently, pathologists determined top ten list of scientifically and economically important plant pathogens including plant parasitic nematodes, fungi, viruses, and bacteria (Jones et al. 2013; Mansfield et al. 2012; Scholthof et al. 2011; Dean et al. 2012) (Table 13.1).

The correct identification and quantification of pathogen causing disease is of major importance in plant health monitoring. However, the detection of plant pathogens based on traditional laboratory techniques such as cultural techniques and microscopy is time-consuming and requires complex sample handling and specialized skills. Because of the importance of the damage caused by plant pathogens, many strategies have been widely developed for diagnosing plant pathogens with a high degree of specificity and sensitivity including DNA-based methods and immunological techniques (Kashyap et al. 2016). In spite of these advantages, most of these technologies have some limitations in detecting pathogens and cannot be applied directly on site detection (Kashyap et al. 2016). Another limitation is related to the high price of some molecular biology reagents, such as primers and enzymes.

By 2050, the population growth is expected to increase from 6 billion to 9 billion, and the global demand for food is expected to grow over the next 40 years due to climate change; limited natural resources such as soil fertility, water, and land; and environmental issues such as the excessive accumulation of pesticides in agri-

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Table 13.1 Top ten important pathogens (nematode, bacteria, virus, and fungi)

S.No.	Nematode (Jones et al. 2013)	Bacteria (Mansfield et al. 2012)	Virus (Scholthof et al. 2011)	Fungi (Dean et al. 2012)
1	*Meloidogyne* spp.	*Pseudomonas syringae*	*Tobacco mosaic virus*	*Magnaporthe oryzae*
2	*Heterodera* spp., *Globodera* spp.	*Ralstonia solanacearum*	*Tomato spotted wilt*	*Botrytis cinerea*
3	*Pratylenchus* spp.	*Agrobacterium tumefaciens*	*Tomato yellow leaf curl*	*Puccinia* spp.
4	*Radopholus Similis*	*Xanthomonas campestris*	*Cucumber mosaic*	*Fusarium graminearum*
5	*Ditylenchus dipsaci*	*Xanthomonas campestris*	*Potato virus Y*	*Fusarium oxysporum*
6	*Bursaphelenchus xylophilus*	*Xanthomonas axonopodis*	*Cauliflower mosaic*	*Blumeria graminis*
7	*Rotylenchulus reniformis*	*Erwinia amylovora*	*African cassava mosaic*	*Mycosphaerella graminicola*
8	*Xiphinema index*	*Xylella fastidiosa*	*Plum pox*	*Colletotrichum* spp.
9	*Nacobbus aberrans*	*Dickeya*	*Brome mosaic*	*Ustilago maydis*
10	*Aphelenchoides besseyi*	*Pectobacterium carotovorum*	*Potato virus x*	*Melampsora lini*

cultural soils (Chen and Yada 2011). Traditional plant protection strategies such as integrated pest management often prove insufficient, and application of chemical pesticides leads to various environmental and human health issues. In addition, the resistance of some plant pathogens and pests against the indiscriminate use of pesticides is rapidly becoming a serious problem (Sangeetha et al. 2017a). To preserve biodiversity, there is an urgent need to achieve pest and disease management by alternate strategies such as nanotechnology. “Nano” word originated from Latin word, which means dwarf. According to International Standards Organization (ISO), nanomaterial is defined as a material with an external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale (1–100 nm). Then the nanotechnology was defined as the science of materials and devices whose structures and constituents demonstrate novel and considerably altered physical, chemical, and biological phenomena due to their nanoscale size (Bhatia 2016). Recently, nanotechnology in agriculture has been increasingly applied to promote food security, food safety, and food production throughout the world (Sangeetha et al. 2017b). In agriculture, nanotechnology has a broad range of applications including nanoparticles (NPs) to manage different plant diseases and pests (Sangeetha et al. 2017b).

Nano-based materials have been widely used to increase the efficacy of pesticides during their applications by allowing minor doses to be used (Abd-Elsalam 2013). In agriculture, particularly, numerous publications and patents have shown the potential benefits of nanotechnological strategies for disease management and crop productivity (Prasad et al. 2012; Parisi et al. 2015; Kah and Hofmann 2014; Mishra and Singh 2016), such as nanofertilizer for enhanced crop productivity, nanopesticides for pest and plant disease management, and nanosensors for detection of plant pathogens and soil monitoring (Barik et al. 2008; Wilson et al. 2008; Oliveira et al. 2015; Prasad et al. 2014, 2017a, b, c; Bhattacharyya et al. 2016; Ismail et al. 2017; Gupta et al. 2018). In this review, we highlighted the potential of nanotechnology applications in the control of plant diseases through the use of metallic nanoparticles and nanosensors for the detection of pests and plant pathogens in different agricultural situations.

13.2 Nanotechnology in Agriculture

With the advancement of industrialization and urbanization, the use of cutting-edge technologies such as nanotechnology and new materials to promote the original innovation of agricultural science and technology is conducive to the sustainable development of agriculture. Recently, nanotechnology has been applied in agriculture and shows good prospects. The development of nanotechnology has led to many new disciplines related to nanometers, such as nanomedicine, nanochemistry, nanoelectronics, nanomaterials, nanobiology, etc., which naturally gave birth to nano-agriculture. Conceptually, nanotechnology agriculture is a material in the field of nanotechnology and agriculture, scientific innovation, and agricultural application of nanomaterial research. Indeed, the application and potency of nanotechnology in agriculture seem to be promising; however, some technical and economic issues should be dealt with. Nanotechnology enables to produce higher yields with lower input costs by streamlining agricultural management and by this way reducing waste and labor costs (increase the level of production at low cost) (Sheykhbaglou et al. 2010). Nevertheless, there is a need to deal with these aims adopting precision farming practices and effective application of nanotechnology (Chowdappa and Gowda 2013).

In fact, the application of integrated innovation and nanotechnology in agriculture is not new. In order to overcome food security, resource scarcity, and environmental issues, in 2000, the National Nanotechnology Innovation Initiative officially incorporated agricultural nanotechnology into the research agenda. In 2003, the US Department of Agriculture launched a special study on the application of nanotechnology in agriculture and food. In the past 10 years, the European Union, Brazil, Canada, China, and other major agricultural countries have strengthened research and development in nano-agriculture. FAO, WHO, and other international organizations are also very concerned about nanotechnology for global agriculture and food security. Practically, nanotechnology can be used to improve the production process and to improve the effectiveness of the new varieties in organic crop cultivation. It is also adopted to enjoy targeted delivery and controlled release of functional nanomaterials in order to improve chemical fertilizers and pesticides and for effective use of other agricultural inputs such as veterinary drugs and animal feeds to reduce residues and pollution. In addition, it can also benefit from the use of nanotechnology to improve monitoring and diagnostic analysis capabilities for major agricultural "epidemics," food safety, and food nutrition. At a quality scale, the content, flavor, and external quality can be improved using nanomaterials in the agricultural production process.

Applications of nanotechnology in the research filed related to agriculture have made important strides especially in reducing pesticide particles from the traditional 5 microns to 100 nm. The small size effect can reduce the shedding of pesticides on the foliage and advance the application of pesticides. Concurrently, the use of nanomaterials to load pesticide particles can achieve controlled release through microencapsulation technology according to the aging characteristics of crop control and extend the duration of application, thus reducing the number of pesticide application times, thereby avoiding food safety issues caused by pesticide abuse. Nanotechnology can also enhance the water solubility and dispensability of poorly soluble pesticides and reduce the use of organic solvents in pesticide formulations (Zhao 2014). The process of nanomaterial production involves cut-edge technologies to engineer nanostructure devices with minimum dimensions less than 100 nm. Several studies report that nanoprocessing methods are based on an unprecedented growth of knowledge and deep understanding of the characteristics, properties, and their integration with engineered nanomaterials into multifunctional devices (Schäffer et al. 2000; Lee et al. 2003; Smith et al. 2003; Ginger et al. 2004; Gates et al. 2005; Biswas et al. 2006; Acharya et al. 2008; Ariga et al. 2007, 2008, 2010, 2011; Rogers and Lee 2008; Sakakibara et al. 2011; Ando et al. 2010; Kraemer et al. 2009; Li et al. 2009; Mailly 2009; Marrian and Tennant 2009; Schmid et al. 2009; Yaman et al. 2011).

Based on the ultrasmall size of nanomaterials, nanotechnology could be less harmful to the environment and human health; however, there are studies showing the potential health hazards and toxic effects since these nanomaterials when entered into a human body lead to tissue damage by the easiness to reach all vital organs.

13.3 Nanotechnology for Plant Disease Detection

A rapid and reliable diagnostic test to identify and quantify pathogens in samples is an essential step toward managing plant disease. However, the diagnostic of these pathogens based on traditional method is time-consuming, lacks high sensitivity, and requires specialized skills. Therefore, molecular tools have been widely used for diagnosing plant diseases including DNA-based molecular diagnostics (PCR with species-specific primers, quantitative PCR, sequencing, etc.) and immunoassays for the detection of pathogen nucleic acid and proteins extracted from infected plants (Lopez et al. 2003; Khater et al. 2017). Several previous studies addressed pathogen detection using immunoassays (serological assays) and nucleic acid-based methods (Nolasco et al. 2002; Anwar Haq et al. 2003; Teixeira et al. 2005; Lacava et al. 2006; Li et al. 2006; Ruiz-Ruiz et al. 2009). Despite these advantages, molecular techniques have some limitations in detecting pathogens directly in the field. Moreover, another limitation is related to false-negative results which can be produced by PCR failure due to degraded DNA, presence of inhibitors, or other reasons (Louws et al. 1999; Lopez et al. 2003; Waeyenberge et al. 2009; Martinelli et al. 2015). To overcome such limitations, there is an urgent need for accurate and early detection of pathogens with the help of effective application of nanotechnology in agriculture. An early diagnosis of disease plays a significant role in health monitoring. It allows to reduce the risk of disease transmission and spread, prevent introduction of new pathogens at country border, and minimize crop loss (Strange and Scott 2005; Miller et al. 2009). Several previous studies addressed rapid diagnostic tools for the detection of plant pathogens using quantum dots, nanoparticles, nanosensors, and nano-based kits (Khiyami et al. 2014; Fan et al. 2003; Arya et al. 2005; Yao et al. 2009; André Lévesque 2001; Duhan et al. 2017; Abd-Elsalam and Prasad 2018). Quantum dots have been widely used to detect different plant pathogens (Arya et al. 2005; Khiyami et al. 2014). Quantum dots are semiconductor nanoparticles which are rapid than organic fluorescent dyes used as proteins for visual detection or markers on nucleic acids (Duhan et al. 2017). A method was developed to identify vector of beet necrotic yellow vein virus using quantum dots-fluorescence resonance energy (Safarpor et al. 2012). Rad et al. (2012) used the same method “Quantum dots-fluorescence resonance energy transfer based sensors” to detect *Phytoplasma aurantifolia* on lime with high sensitivity.

In the last years, biosensors have been widely used as diagnostic tools in food to improve pathogen and environmental analyses. They allow to improve pathogen detection techniques in different crop systems (Khater et al. 2017). Biosensor strategies are applied through different receptors including DNA probe and antibody-based biosensors (Singh et al. 2013; Khiyami et al. 2014). Moreover, nanosensors can detect rapidly the presence of plant viruses, bacteria, and crop pathogens with precise quantification (Otles and Yalcin 2010; Brock et al. 2011; Khater et al. 2017). Several previous studies addressed plant disease detection such as fungi (Chartuprayoon et al. 2010), virus (Yao et al. 2009), and bacteria (Boonham et al. 2008) using nanoparticles. Recently, gold nanoparticle (AuNP) aggregation-based DNA analyses have widely been used for the detection of plant pathogens' DNA (Khater et al. 2017). Various studies reported the application of DNA-based biosensors for diagnosing plant pathogens with high degree of sensitivity and specificity (Table 13.2)

13.4 Application of Nanotechnology for Controlling Pest and Pathogens

Nanotechnology has been provisionally defined as relating to materials, systems, and processes which operate at a scale of 100 nm or less. A nanometer is one billionth of a meter. Overall nano refers to a size scale between 1 and 100 nm. For comparison, the wavelength of visible light is between 400 and 700 nm. A leukocyte has the size of 10,000 nm, a bacteria 1000–10,000 nm, virus 75–100 nm, protein

Table 13.2 DNA-based biosensors used for plant pathogen detection

Assay format detection	Sensing plant disease	References
AuNP aggregation-based DNA	*Pseudomonas syringae*	Vaseghi et al. (2013)
	Fusarium oxysporum	Wee et al. (2015)
	Botrytis cinerea	
Fluorescent approach in microfluidics based on silver NPs	*Phytophthora* species	Schwenkbier et al. (2015)
Turbidity-based microfluidic system	*Cymbidium mosaic virus*	Chang et al. (2013)
DNA microarray technology	*Botrytis cinerea* *Botrytis squamosa*	Wang and Li (2007)
	Cucumber mosaic virus	Zhang et al. (2005)
	Potato viruses	Boonham et al. (2003), Bystricka et al. (2003)
	Cucumber mosaic virus	Deyoung et al. (2005)
Electrochemiluminescence-based DNA	*Banana bunchy top virus* *Banana streak virus* *Papaya leaf curl virus*	Tang et al. (2007)

5–50 nm, deoxyribonucleic acid (DNA) ~2 nm (width), and an atom ~0.1 nm. Nanotechnology considers the topics with viruses and other pathogens scale. So, it has the high potential to identify and eliminate pathogens (Predicala 2009; Prasanna 2007). The utilization of nanomaterials as a pesticide to control and prevent plant disease is an effective tool that may be adopted, meaning several methods and the evident methods would be the direct application in soil and on seeds and foliage (Khan and Rizvi 2014). The mode of action of nanomaterials for direct application is the same as conventional pesticides. However, the physicochemical characteristics are strictly different from conventional pesticides to formulated ones as nanomaterials. Based on this finding, the effect on pathogens (bacteria, fungi, and virus) evolved more attention to test and evaluate the effect on these nanopesticides. To elucidate this particularity, Khan and Rizvi (2014) reported that the use of silver at its normal form has no effect on the microorganisms. However, a formulated silver at nanoform was shown to be toxic on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumonia*. The possible explanation according to the same study was that the nanoform of silver has a definite effect on the inhibition of colonization of bacteria and fungi.

In recent years, several plant pathologists harnessed the benefits of nanomaterials for controlling pests and plant diseases. A number of exciting results were obtained for controlling plant pests, especially nematodes, insects, bacteria, and fungi by application of different techniques of nanomaterials.

13.4.1 Plant-Parasitic Nematodes

Plant-parasitic nematodes (PPNs) are one of the main biotic causes of plant stress and yield loss in world agriculture (Nicol and Rivoal 2008). Global yield losses due to damage by plant-parasitic nematodes are estimated at 7%, representing annual monetary losses of 5.8 billon US $ (Sasser and Freckman 1987). Many attempts are being developed for managing PPNs, including chemical control, cultural practices, and development of resistant varieties which cause reduction of nematode population densities to levels below damage thresholds (McSorley and Duncan 1995). Since the1950s, synthetic pesticides have been traditionally used by farmers for managing nematodes. However, the indiscriminate and unsafe use of pesticides is hazardous to environment and agro-ecosystems. To expand the choice of effective management methods, the search for benefits of nanomaterials against PPNs is gaining interest.

13.4.1.1 Nanosilver-Silicon

Silver nanoparticle (AgNP) has shown a great potential as an effective nematicide for controlling PPNs (Roh et al. 2009), and its nematicide effect is related with induction of oxidative stress in the cells of PPNs and disrupting cellular mechanism of membrane permeability and ATP synthesis (Ahamed et al. 2010; Lim et al. 2012). Cromwell et al. (2014) evaluated the nematicidal effect of silver nanoparticles (AgNPs) in laboratory and field experiments. They concluded that high application doses ($\geq$90.4 mg/m^2) of AgNP have been found to be effective in reducing the number of juveniles of *Meloidogyne graminis* in turfgrass. In addition, several researchers reported the effect of high concentrations of AgNP in reducing the entomopathogenic nematodes including *Steinernema abbasi*, *S. arenarium*, and *Heterorhabditis indica* (Taha and Shady 2016). Nasar (2016) evaluated the efficacy of Ag-nanoformulations of extracts of *Urtica urens* against root-knot nematodes (*Meloidogyne incognita*), and he revealed that the effect of petroleum ether extract and its Ag nanoparticles offers a satisfactory and environmentally friendly way of reducing *Meloidogyne incognita.* Abdellatif et al. (2016) reported that the use of green silver nanoparticles reduced significantly root galls caused by root-knot nematode (*Meloidogyne javanica*) in eggplant. They concluded that 12.75 mg 100 mL^{-1} of green silver nanoparticles was effective for controlling *M. javanica* without any phytotoxicity in eggplants. Moreover, Taha (2016) evaluated the effect of silver nanoparticles (AgNPs) against *Meloidogyne incognita* in screen house and laboratory. The same author concluded that the nematode populations of *M. incognita* associated with tomato were lower in soil treated with the concentration (1500 ppm) of AgNP. Finally, Goswami (1993) showed that the nematode populations of *Meloidogyne incognita* associated with cowpeas were lower in soil amended with *Azadirachta indica* than in dried and autoclaved soils

13.4.1.2 Plant Virus Nanoparticle (PVN)

Recently, several biological nanoparticles based on plant virus have been used for controlling PPNs. This method allows to deliver more pesticides to the roots by using plant virus and shows promising results for controlling crop diseases including nematodes (Chariou and Steinmetz 2017). Cao et al. (2015) developed a new method for increasing the mobility of Abamectin (biological pesticide) within the soil by loading it into a *Red clover necrotic mosaic virus* (RCNMV)-derived plant virus nanoparticle (PVN), and they showed egg masses and root galling caused by *Meloidogyne hapla* in tomato seedlings. Moreover, Charioui and Steinmetz (2017) evaluated the effect of a nematicide called crystal viol that encapsulated a *Tobacco mild green mosaic virus* (TMGMV)-derived plant virus nanoparticle. The authors showed that the virus allowed to obtain more diffusion of the pesticide to the root level, which causes a reduction of nematodes.

13.4.1.3 Iron Nanoparticles

Iron nanoparticles are a prominent example among nanomaterials due to their wide scope of application in health care, wastewater, medicine, agriculture and food, and energy (Ali et al. 2016). Iron NPs (FeNPs) were used in medicine and biology due to their strong magnetic properties including the magnetic guidance for drug delivery and separation of cells and biological products (Estelrich et al. 2015). Recently, Sharma et al. (2017) evaluated the potential of FeNPs as nematicides against *Meloidogyne incognita* associated with Okra (*Abelmoschus esculentus*), and they concluded that the FeNPs reduced significantly the number of *M. incognita* in Okra due to their high reactivity.

13.4.1.4 Gold Nanoparticles (AuNPs)

For centuries, gold nanoparticles have found their importance as important components for biomedical applications (Yeh et al. 2012). The range of application of the AuNPs has been growing rapidly and includes electronics, photodynamic therapy, therapeutic agent delivery, sensors, probes, diagnostics, and catalysis (Huang et al. 2007; Stuchinskaya et al. 2011; Brown et al. 2010; Ali et al. 2012; Perrault and Chan 2010; Peng et al. 2009; Thompson 2007). Recently, Kucharska et al. (2011) evaluated the effect of gold nanoparticles on the mortality of *Steinernema feltiae* (entomopathogenic nematodes), and they concluded that the concentration of AuNP (5 ppm) caused 78% mortality of nematode after 5 days of experiment.

13.4.2 Insect Pest

Nanoparticles have a great potential for the management and control of pests in modern agriculture. Several previous studies addressed the applications of different nanoparticles (NPs) for controlling insect pests (Yasur and Rani 2015; Murugan et al. 2016; Buhroo et al. 2017). Yang et al. (2009) reported that garlic essential oil with polyethylene glycol-coated nanoparticles reduced significantly the number of *Tribolium castaneum*. Stadler et al. (2010) tested the effect of nanostructured alumina on two insect pests (*Rhyzoperthadominica* and *Sitophilus oryzae* L.). The same authors concluded that after 3 days of exposure, the mortality of both insects was significantly higher. Debnath et al. (2011) concluded that amorphous silica nanoparticles were found to be very effective and can be used as insecticides against rice weevil *Sitophilus oryzae*. Guan et al. (2008) developed a new photodegradable insecticide based on nanoparticles. Various studies reported the application of different nanocomposites and nanomaterials used against insect pests (Table 13.3).

13.4.3 Effect of Nanoparticles on Bacteria

Even though nanotechnology has many applications, cytotoxicity remains the potential concern currently (Chatterjee et al. 2011). The antibacterial effects of nanomaterials on several species of bacteria were confirmed (Fu et al. 2005; Prasad et al. 2016; Aziz et al. 2014, 2015), but the mechanism of action is still not yet understood. Indeed, Warheit (2008) reported that different factors (synthesis, shape, size, composition, and stabilizer) can lead to different results even for close nanosuspensions. Jayaseelan et al. (2012) reported that zinc as a nanoparticle has an antibacterial activity on *P. aeruginosa*, and the maximum diameter of inhibition obtained in this study was 22 mm by using 25 ng mL^{-1} ZnO as a nanoparticle. The nanomaterials composed of silver and PVP (polyvinylpyrrolidone) showed a good control of three pathogenic bacteria, *S. aureus* (Gram-positive), *E. coli* (Gram-negative), and *P. aeruginosa* (Gram-negative), and one beneficial bacteria *Bacillus subtilis* (Bryaskova et al. 2011). The same effect was observed after using CuO as a nanomaterial (Azam et al. 2012; Guzman et al. 2009). Khan and Rizvi (2014) reported that nanomaterials were dependent on concentration, physiology, metabolism, intracellular permeability, and the type of microbial cell.

13.4.4 Effect of Nanoparticles on Fungi

Fungicidal activity of nanomaterials was reported on fungi (Bryaskova et al. 2011; Sharma et al. 2009; Sondi and Salopek-Sondi 2004; Aziz et al. 2016). Singh et al. (2013) have found among 15 micronutrients in their nanoforms, only $CuSO_4$ and

Table 13.3 List of different nanocomposites and nanomaterials used against insect pests

Metal	Pest(s)	References
Synthesis of bionanoparticles (NPs) using plant extract		
AgNPs—Aqueous leaf extracts of *Euphorbia prostrata*	*Sitophilus oryzae*	Zahir et al. (2012)
NPs loaded with garlic essential oil	*Tribolium castaneum* (Herbst)	Yang et al. (2009)
Silver NPs—Aqueous leaf extract of *Tinospora cordifolia*	*Pediculus humanus*, *Anopheles subpictus*	Jayaseelan et al. (2011)
Silver—*Avicennia marina*	*Sitophilus oryzae*	Sankar and Abideen (2015)
PCL nanospheres—*Zanthoxylum rhoifolium*	*Bemisia tabaci*	Christofoli et al. (2015)
Polyethylene glycol NPs loaded with garlic essential oil	*Tribolium castaneum*	Yan et al. (2009)
NPs loaded with neem (*Azadirachta indica*) extracts	*Plutella xylostella*	Forim et al. (2013)
Chemical nanomaterials		
CdS nanoparticle, Nano-Ag, Nano-TiO_2	*Spodoptera litura* larvae	Chakravarthy et al. (2012)
Nanosilica	*Spodoptera littoralis*	El Bendary and El Halaly (2013)
Ag and Zn particles	*Aphis nerii*	Rouhani et al. (2012)
SNPs	*Spodoptera litura* larvae	Debnath et al. (2012)
AgNPs	*Acheta domesticus*	Louder (2015)
Nanosulfur	*Tetranychus urticae*	Gopal et al. (2012)
Aluminum oxide NPs	*Sitophilus oryzae*	Goswami et al. (2010)
Silica nanoparticles	*Spodoptera littoralis* Boisd.	Borei et al. (2014)
Silica nanoparticles	*Sitophilus oryzae*, *Lipaphis pseudobrassicae*	Nitai (2012)
Silica nanoparticles	*Spodoptera littoralis*	El Helaly et al. (2016)
DNA-tagged nanogold	*Spodoptera litura*	Chakravarthy et al. (2012)
Nano-Ca	*Bactrocera dorsalis*	Christenson and Foote (1960)
Calcium carbonte NPs	*Aonidiella aurantii* and *Bactrocera dorsalis*	Hua et al. (2015)
SiO_2 NPs	*Sitophilus oryzae*	Goswami et al. (2010)
SNPs	*Sitophilus oryzae*	Debnath et al. (2011)
Permethrin NPs	*Culex quinquefasciatus*	Anjali et al. (2010)
Nano-Al_2O_3 dusts	*Sitophilus oryzae* and *Rhyzopertha dominica*	Stadler et al. (2010), Buteler et al. (2015)
TiO_2 NPs	*Hippobosca maculata* and *Bovicola ovis*	Velayutham et al. (2012)

(continued)

Table 13.3 (continued)

Metal	Pest(s)	References
Bionanomaterials		
Chitosan nanoparticles	*Spodoptera litura*	Arvind Bharani et al. (2014)
Chitosan nanoparticles	*Callosobruchus maculatus*	Sahab et al. (2015)
Nano-diatomaceous earth	*Tribolium confusum* and *T. castaneum*	Sabbour and Abd El Aziz (2015)
Novaluron nanoparticles	*Spodoptera littoralis*	Elek et al. (2010)
Pheromone nanogels		
A nanogel prepared from the pheromone methyl eugenol	*Bactrocera dorsalis*	Baghat et al. (2013)
Carum copticum essential oil-loaded myristic acid-chitosan nanogels	*Sitophilus granarius*, *Tribolium confusum*	Ziaee et al. (2014)
Cellulose acetate nanofibers	*Lobesia botrana*	Bansal et al. (2012)
Cypermethrin nanofibers	*Grapholita molesta*	Czarnobai De Jorge et al. (2017)

$Na_2B_4O_7$ were found to have fungicidal activity on rust disease of peas (*Uromyces* sp.). In sunflower crop, the damping off and charcoal diseases were suppressed by nanoforms of manganese and zinc (Abd El-Hai et al. 2009). Other nanoparticles have been reported to cause a deformation in the hyphae of *B. cinerea* and prevented the development of conidiophores and conidia in *P. expansum* which eventually led to the death of fungal hyphae (Prasad et al. 2017b). Krishnaraj et al. (2012) tested the effect of silver nanoparticles on plant pathogenic fungi, *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *B. cinerea*, and *Curvularia lunata*, and found that 15 mgL^{-1} concentration has a fungicidal activity on all the tested pathogens.

13.5 Future Prospects and Conclusion

In recent decades, the fast development of nanotechnology has paved the way for developing new approaches for various agricultural problems including plant protection and detection of diseases. Indeed, with a wide range of applications of nanotechnology in the future, we might expect nanoparticles will be extensively used for the management and control of plant pathogens. Nanomaterials can be used in plant protection and management of farm practices based on their small size.

Despite the wide application scope of nanotechnology and its benefits in plant disease management, there are several challenges in the field, about the potential risk in the use of nanoparticles in managing plant pathogens which is required to be solved prior to use in agricultural systems. The most pressing problem is the phytotoxicity of nanomaterials in the plant pathogen management which needs to be ascertained during plant growth.

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